Rui Amado Figueiredo Santos

SYNTHESIS OF BIOCOMPATIBLE HYDROGELS FOR DRUG ENCAPSULATION

Dissertation guided by Jorge F.J. Coelho, PhD, and Arménio C. Serra, PhD, and presented to the Faculty of Sciences and Technology of the University of Coimbra to obtain a Master's degree in Biomedical Engineering

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Universidade de Coimbra

FCTUC FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

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Rui Amado

"We cannot reason ourselves out of our basic irrationality.
All we can do is learn the art of being irrational in a reasonable way."

Aldous Huxley, in Island

Abstract

This work comprises the formulation of biocompatible hydrogels for drug encapsulation. Hydrogels are polymers with a three-dimensional matrix which can absorb a large volume of water due to its hydrophilic functional groups, being widely used in biomedical applications, such as drug delivery systems. Dextran was the chosen polymer to prepare the hydrogels and, on behalf of being a polysaccharide, presents excellent properties such as non-toxicity, water solubility, and biocompatibility. The course of studies encompasses the chemical modification of dextran by means of cross-linking agents and the study of the swelling behaviour of the prepared hydrogels, in order to produce a suitable three-dimensional network which allows the encapsulation of the drug 5-Fluorouracil and a monitoring of its releasing profile.

Key words: Hydrogel; Dextran; Polysaccharide; Drug encapsulation; Controlled release; 5-Fluorouracil;

Resumo

Este trabalho centra-se na preparação de hidrogéis biocompatíveis para encapsulação de fármacos. Os hidrogéis são polímeros com redes tridimensionais que conseguem absorver grandes volumes de água devido aos seus grupos funcionais hidrofílicos, sendo amplamente utilizados em aplicações biomédicas tais como sistemas de libertação de fármaco. O dextrano foi o polímero escolhido para a preparação dos hidrogéis e, tendo em conta o facto de ser um polissacarídeo, demonstra excelentes propriedades como não-toxicidade, solubilidade em água e biocompatibilidade. O curso dos estudos focaliza-se na modificação química do dextrano por meios de agentes reticulantes para a formação de hidrogéis e estudo das suas capacidades de inchaço, de forma a produzir uma matriz tridimensional apropriada para a encapsulação do fármaco 5-Flourouracilo e monitorizar o seu perfil de libertação.

Palavras-chave: Hidrogel; Dextrano; Polissacarídeo; Encapsulação de fármaco; Libertação controlada; 5-Fluorouracilo;

List of Abbreviations and Acronyms

5-Fu – 5-Fluororacil

ATR – Attenuated Total Reflectance

Bis-MPA – 2,2-bis-(hydroxymethyl)-propionic acid

BTCA – 1,2,3,4-butanetetracarboxylic acid

CL-X – Cross-linking agent X

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic acid

FTIR – Fourier Transform Infrared Spectroscopy

IR - Infrared

PLA – Polylactic acid

PVA – Poly(vinyl alcohol)

RNA – Ribonucleic acid

SEM – Scanning Electron Microscopy

UV-Vis – Ultraviolet-Visible

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1 Introduction

Introductory Observation

The presented work comprised the development of dextran-based biodegradable materials for controlled drug releasing. In the process of the studies it was noticeable that the established method, in spite of requiring certain improvements, could be applicable in a wide range of purposes. Nothing similar was found through an extensive literature review, thus providing an innovative perspective to the work. Due to a scarcity of time, this work was not further developed in practical terms, specifically, in concern to the drug-releasing results, which were not well settled at the time. Since a final evaluation of the whole process and assessment of reliable results is not yet attainable, and, if in the future the required improvements may be settled and it is possible to achieve a process of intellectual protection rights, it was decided that the most critical parts of the work would deliberately remain confidential. These critical parts introduce the major differences in what is described, however, it is considered that their absence does not compromise any critical analysis or evaluation, which should be focused on errors and inaccuracies always depicted in works of this nature.

1.1 Polymers

1.1.1 Basic Concepts

The word polymer derives from the classical Greek *poly* (meaning 'many') and *meres* (meaning 'parts') and is used to define a large molecule (macromolecule) composed by the repetition of smaller chemical units. These smaller chemical units are entitled monomers. A polymer can be constituted by one or several types of different monomers. In Figure 1.1 is presented the example of the monomer styrene - after cleavage of the double bond, the monomers can be linked together and result in the polymer polystyrene, with the repeating unit being inside square brackets.

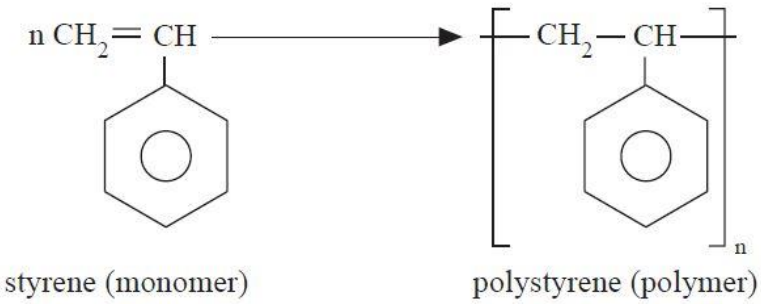

Figure 1.1 – Monomer styrene and polymer polystyrene. [1]

Although the repeating unit goes through an electron rearrangement, it still retains identical atoms and similar relative positions. Polymerization occurs by a sequential reaction, as the repeating units are bonded together and progressively form a larger molecule.

The monomeric structure employed to obtain the polymer is referred as the structural unit, and some polymers result from the reaction of a single type of monomer, as others are a result of the reaction of two or more monomers (copolymer). The n designation represents the number of repeating units linked together in the chain and is known as degree of polymerization, as it represents the length of the polymer chain. [1][2]

1.1.2 Classification Of Polymers

Regarding their occurrence, polymers can be classified as: natural (known as biopolymers); synthetic (fully synthesized by man) and semi-synthetic (chemically modified natural polymers). They can be classified by other properties, such as structure, polymerization mechanism, preparation techniques or thermal behaviour.

Based on their structure, polymers can be linear, branched or cross-linked. In Figure 1.2 are represented - (a) linear polymer, that consists of a long chain of monomers; (b) branched polymer, which has branches bonded covalently to the main chain; (c) cross-linked polymer, having monomers which are covalently bonded to monomers of another chain, thus creating a three-dimensional network.

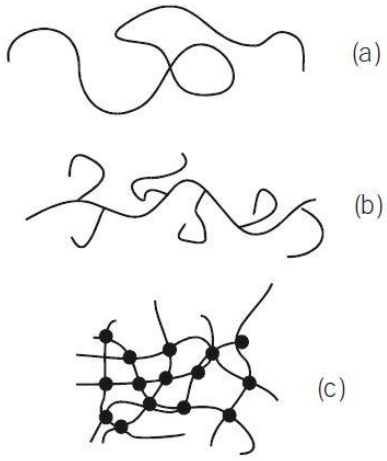

Figure 1.2 – Structure of polymers: (a) linear polymer; (b) branched polymer; (c) cross-linked polymer. [2]

Polymers can also be classified, as seen in Figure 1.3, as: (a) homopolymers, consisting of monomers of the same type, or as copolymers, which have different repeating units. Copolymers can be: (b) random, where the different repeating units are distributed randomly across the chain; (c) alternating, where the chains have alternating sequences of the different monomers; (d) block copolymers, where long sequences of the same monomer are followed by long sequences of another; and (e)

graft copolymers, which have branches of one monomer grafted to chains of another type. [1][2]

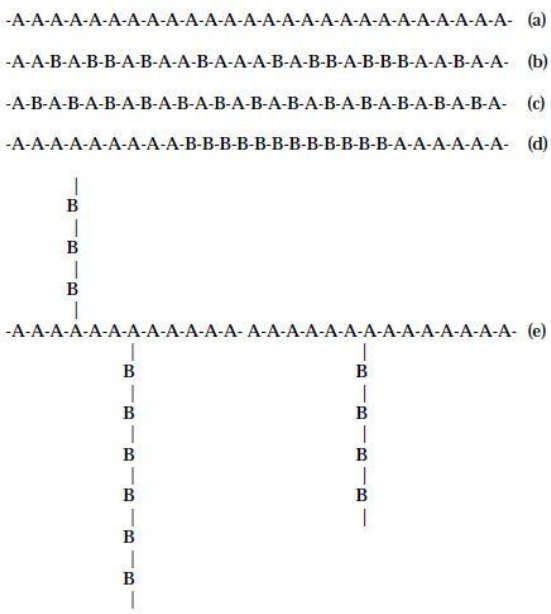

Figure 1.3 – Classification of polymers: (a) homopolymer; (b) random copolymer; (c) alternating copolymer; (d) block copolymer; (e) graft copolymer. [2]

1.2 Polysaccharides

Polysaccharides are the form of natural occurrence of carbohydrates, establishing high molecular weight polymers and standing as the fundamental macromolecules of life. They comprise two main purposes - providing energy storage and establishment of extracellular structural units. They embrace several origins including algae (*e.g.* alginate, agar and carrageenan), plants (*e.g.* cellulose, pectin and guar gum), microbial (*e.g.* dextran and xanthan gum) and animal (*e.g.* heparin, chondroitin, chitin). [3][4]

In general, polysaccharides are polymeric carbohydrate structures made from repeating units linked together by glycosidic bonds, with their structures being often linear, however they might present several degrees of branching. This

branching ability distinguishes polysaccharides from proteins and nucleic acids, which occur only as linear polymers. [5]

They have the general formula $C_x(H_2O)_y$, where y stands between 200 and 2500 and x=y-1. By considering that the common structures usually are six-carbon monosaccharides, the formula stands as $(C_6H_{10}O_5)_n$, where n is between 40 and 3000. [6]

The basic composition of polysaccharides is either made by one type of repeating unit, or two or more alternating units linked together by glycosidic bonds, hence, one can find two main types of polysaccharides, as seen in Figure 1.4 - homopolysaccharides, which are made up by a single type of monomer; and heteropolysaccharides, which have two or more types of monomer units in its composition. [3]

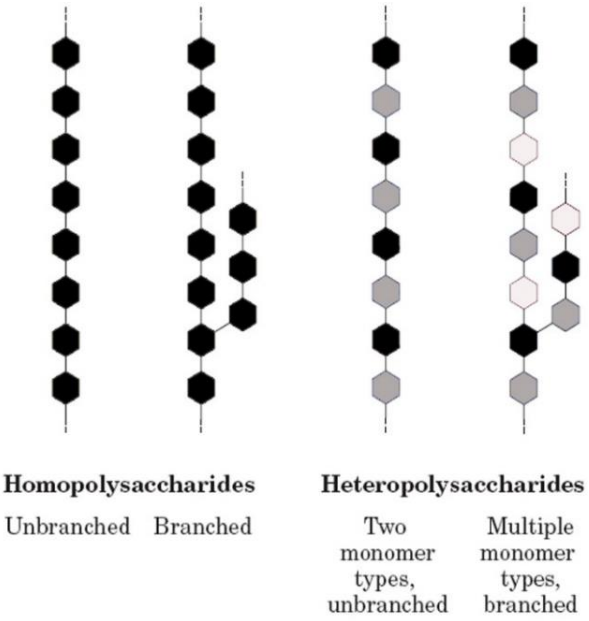

Figure 1.4 – Polysaccharide basic composition: unbranched and branched homopolysaccharides; unbranched and branched heteropolysaccharides. [3]

The majority of homopolysaccharides act as energy storage for biological processes, such as starch and glycogens, and can work as structural elements in plant cell walls and animal exoskeletons, such as cellulose and chitin. Heteropolysaccharides offer extracellular support for microorganisms like bacteria

and animal tissue, where the extracellular space is filled with heteropolysaccharides and creates a matrix that holds together individual cells and provides protection, shape and support to those cells. [3]

Naturally occurring polysaccharides are diverse in their physicochemical properties since multiple structures exist and the chemical composition largely differs. Frequently, the molecular weight is not well-defined due to the mechanism of polymer formation, which is a natural process of polymerization of monomeric units catalyzed by certain enzymes in different organisms. [3][4]

Also designated as glycans, attributable to the glycosidic bonds, the classification of polysaccharides is centred on the existing monosaccharides, the length of chains, the degree of branching, linkage mode and sequences between chains as well as the anomeric configuration (α or β) of those linkages, ring sizes (furanose or pyranose), absolute configuration (D- or L-), and presence of other substituents such as nucleic acids and proteins that may have influence to determine the functionality and specificity of the polysaccharide. [3]

In Figure 1.5 are represented several examples of average polysaccharide structures.

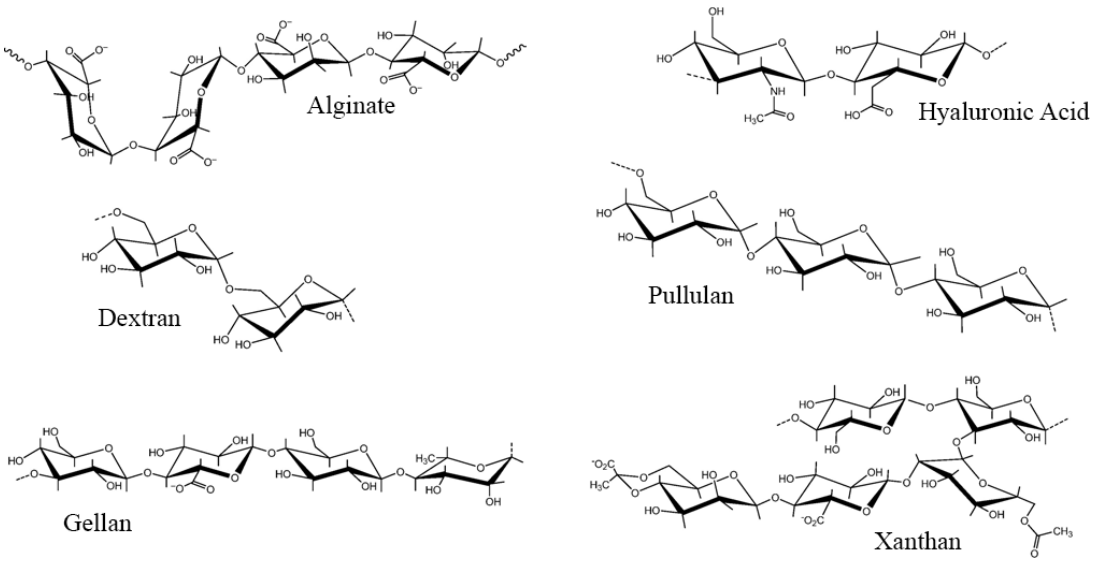

Figure 1.5 – Examples of polysaccharide structures. [26]

The physicochemical properties of polysaccharides depend on structural features such as conformation and intermolecular relations, and the most stable atom arrangement is achieved when it fulfils both intra- and inter- molecular forces. Their conformation is restricted because of the steric limitations on the free rotation of the glycosidic bonds in the sugar molecules. [3]

Carbohydrates containing a great number of hydroxyl groups are hydrophilic and are capable of creating polar surfaces, increasing their interaction with water. The hydroxyl groups are able to interact with two water molecules simultaneously, if they are not interacting with other hydroxyl groups within the molecule. These interactions with neighbouring polysaccharides may result in a reduced hydration, as β-linkages existing at 3- and 4-positions allow a strong inflexible hydrogen bonding, thus reducing hydration and increasing the inflexibility of the structure; on the other hand, α-linkages present at 2-, 3- and 4-positions increase the hydration of the polymer for more flexible linkages. [3]

From a biomedical perspective, polysaccharides have several advantages such as low toxicity, biocompatibility, stability, low-cost, hydrophilic nature and availability of reaction sites for chemical modifications. The chemical functionalizations are attained by using mostly the free carboxyl and hydroxyl groups present in the polysaccharide backbones and are employed to create polysaccharide derivatives with specific tailored properties. [4]

Taking into consideration this susceptibility of polysaccharides for chemical modifications, they are appealing macromolecules for the formulation of targeted drug delivery systems due to their improved pharmacokinetics and pharmacodynamics for small drugs, proteins and enzymes. The physicochemical properties such as molecular weight, structure, and charge, may considerably impact the dynamics of the macromolecule-drug conjugates and the release profile in the desired medium. [3]

Several polysaccharide applications in healthcare are summarized in Table 1.1. [7]

Table 1.1 – Common polysaccharides and their applications in healthcare. [7]

Polysaccharides	Applications
Starch and derivatives, cellulose and derivatives, chitosan, dextran, hyaluronan, pectin, carrageenan	Drug delivery
Chitosan, alginates, bacterial cellulose, hyaluronan	Wound healing
Cellulose, hyaluronan, chitosan, alginates	Tissue engineering (scaffolds and implants)
Heparin, chitosan	Bioactive compounds (anti-microbial, blood anti-clotting, drugs and vaccines)
Hyaluronan, chitosan, aloe	Skin hydration, anti-aging agents, skin protection (antibacterial agents)
Bacterial polysaccharides	Vaccines

1.3 Dextran

Dextran stands as one of the most important polysaccharides used both in medical and industrial fields. Dextrans constitute a family of neutral exocellular bacterial polysaccharides that consist mainly of an α-(1,6)-glucopyranose main chain with variable extents of linkages and branches. The α-(1,6) linkages account for 50 to 97% of all glycosidic bonds, while α-(1,2), α-(1,3) and α-(1,4) linkages commonly connect as branches.

In Figure 1.6 is presented the α-(1,6)-linked main chain of dextran, as well as branching points in 2-, 3- and 4- positions. [8]

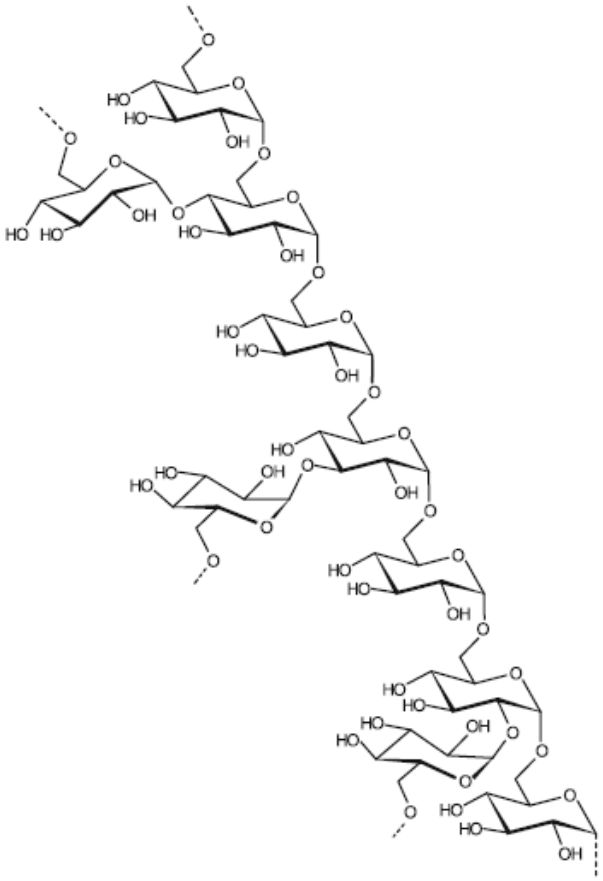

Figure 1.6 – Dextran chain structure. [8]

Several bacterial strains, such as *Lactobacillus*, *Leuconostoc* and *Streptococcus* are able to synthesize dextran, generally from sucrose or other carbohydrate mediums with anhydro-D-glucopyranose units. There are industrial ways of producing dextran, but the majority of the manufacturers still resort to the bacterial procedure presented by Jeanes *et al.*, based on the fermentation of *Leuconostoc mesenteroides* in the presence of sucrose. Furthermore, several medium aspects combined with different microorganisms and polysaccharide concentrations give origin to dextran with different properties and structures, such as degree of branching, relative amount of certain glycosidic bonds, molecular weight, solubility, optical activity and physiological action. [3][8][9]

The product of the microbiological synthesis is termed 'native dextran' and has a molecular weight ranging from 10^7 to 10^8 Da. Its molecular weight can be

reduced by acid hydrolysis, and clinical grade dextrans are obtained from high molecular weight native dextran after partial depolymerization by acid hydrolysis and fractioning. [3]

As result of characteristics like common solubility in water and several other solvents (*e.g.* DMSO, formamide), biocompatibility, and the susceptibility of degrading in certain physical environments, dextran has been applied both in medical and biomedical fields. Additionally, the branched homopolymer structure based on D-glucose units is deprived of relevant imperfections, which is favourable for chemical modification. Moreover, when compared to products derived from starch and cellulose, dextran derivatives may display dissimilar characteristics in the polysaccharide chain and secondary groups, even when the same functional groups are introduced. As a result, multifunctional dextran derivatives can be prepared with tuneable properties, depending on the introduced substituent. [3][8][10]

Contrasting with other polysaccharides such as chitosan, alginate or hyaluronic acid, which maintain various functional groups, dextran only comprises hydroxyl groups. Consequently, the incorporation of other functional groups in the binding sites via chemical modification is appealing, in order to achieve the desirable functionalities. For each repeating unit dextran holds three free hydroxyl groups, with the reactivity decreasing in the order C2>C4>C3. The degree of substitution refers to the amount of replaced hydroxyl groups per unit and it influences the properties of its derivatives. In short, the large number of hydroxyl groups makes dextran suitable for derivatization and subsequent chemical or physical cross-linking. Dextrans also remain stable under mild acidic and basic environments and are subject to enzymatic degradation by dextranase, which exists in mammalian tissues. [11][12][13]

Dextran has been used for selective transport and accounts as a carrier for a wide-ranging of therapeutic agents due to their worthy physico-chemical properties and physiological acceptance. It is applied in fields such as drug targeting, cardiocirculatory time control, stabilization of therapeutic agents, solubilization of drugs, controlled release, among others. For instance, clinical grade dextran is available for replacing moderate blood losses, since the polymer essentially substitutes blood proteins and provides colloid osmotic pressure to flow the fluid from the interstitial space into the plasma. Likewise, some molecular weights of dextran have the feature of improving blood flow by lowering its viscosity and preventing erythrocyte aggregation, being used as artificial colloids. Additionally, dextran is used for the preservation of viable organs and also as an ingredient for ophthalmic formulations. [3]

Outside the medical field, dextran it is often used as an ingredient into several other industries. In food industry, for instance, it is incorporated into bakery products to improve softness, and into ice creams and candy as a stabilizer to improve moisture and maintaining flavour. In cosmetics, dextran is used as a moisturizer and thickener. These are just a few example among many others, showing that dextran is a versatile polymer with applications not only into medical and pharmaceutical industries, but in other industries as well. [8][14]

1.4 Hydrogels

Hydrogels are defined as three-dimensional, cross-linked networks of water-soluble polymers which are able to absorb and hold water or other biofluids within their porous structure. This feature is due to the presence of hydrophilic groups (amino, carboxyl and hydroxyl), providing high water holding capacity. On the other hand, increasing the cross-linking density of the network is followed by a decrease of hydrophilic groups, thus lowering the hydrogel swelling capacity. The

networks are set up by homopolymers or copolymers as a result of the presence of chemical cross-links such as covalent bonds that form tie-points or junctions, or physical cross-links, like entanglements or ionic interactions. [15][16]

Commonly, hydrogels are prepared to have hydrophilic properties by using hydrophilic monomers, however, in order to obtain certain properties for specific applications they require the use of other compounds, such as hydrophobic compounds. [17]

The presence of different functional groups plays an important role in the water holding capacity in hydrogels, so, it is important to analyse which functional groups are present in the structure of the polymeric network in order to acquire more information about the possible final properties of the product. There are several techniques such as infrared spectroscopy, UV-visible spectroscopy, nuclear magnetic resonance or mass spectrometry which make it possible to determine the structure and properties of the hydrogels. [15]

Biocompatibility is a very important characteristic required in a hydrogel and is accomplished mainly due to their high water content and the physico-chemical similarities to the native extracellular matrix, both in terms of composition and mechanical behaviour (predominantly in polysaccharide-based hydrogels). The hydrophilic surface of hydrogels shows a low interfacial free energy when in contact with body fluids, thus lowering the tendency for proteins and cells to adhere to their surfaces. Additionally, their soft and rubbery nature minimizes irritation in the nearby tissues. Biodegradability is ensured via enzymatic cleavage, hydrolytic ways or physicochemical degradation by environmental means (*e.g.* pH, temperature), and the degradation products should not show toxic, as they should be metabolized and excreted without adverse effects. [16][18][19]

1.4.1 Swelling And Absorption Capacity

The swelling behaviour and absorption capacity are among the most important properties of hydrogels when it comes to evaluate their possible use in a wide range of applications. These properties are attributed to the presence of hydrophilic groups such as -OH-, CONH-, -$CONH_2$-, or -SO_3H in the network, and the swelling ratio is characterized by the change in mass when the hydrogel is swollen (water intake) and dried. Several factors may affect this ratio, such as chemical composition, network structure, solvent concentration and quality, cross-linking ratio and other stimuli by the surrounding medium like temperature or pH. [17]

Hydrogels with hydrophilic groups have higher water swelling degree when compared to others with hydrophobic groups, since hydrophobic groups might collapse in the presence of water, lowering the exposure to the water molecules and consequently lowering the swelling ratio. [17]

The swelling process occurs when the solvent penetrates the polymer network, expanding the space between cross-linked points and decreasing the matrix configuration enthalpy value. The network also has an elastic contractive force that attempts to make the network contract. Fundamentally, the osmotic pressure generates the swelling expansion and the network elastic force causes the force of contraction. Once these opposing forces reach equilibrium, a balance takes place between expansion and contraction. [17]

Focusing on the network level, increasing of the cross-linking density results in an increase in hydrophobicity and a decrease in stretchability of the matrix structure, leading to a reduced swelling capacity and reduced diffusion through the hydrogel. [15]

In addition to the regular swelling behaviour, hydrogels can be sensitive to small changes in the surrounding environment, such as changes in temperature, pH,

ionic strength, light, electric field, or presence of electrolytes. These stimuli sensitive hydrogels exhibit variations in their network swelling behaviour according to these changes in external stimuli. [19]

1.4.2 Classification Of Hydrogels

Hydrogels can be classified as from several different points of view [20]:

- Neutral (nonionic) or ionic (cationic or anionic) - considering the nature of their side groups;
- Affine (cross-links are firmly connected) or phantom (cross-links fluctuate) - according to their mechanical properties and cross-link structure;
- Copolymer or homopolymer networks - based on the type of monomeric units employed; or multipolymer interpenetrating networks (formed by two independent cross-linked polymers, thus creating a more complex network);
- Amorphous (chains are disposed randomly), semi-crystalline (chains orderly form crystallites in some areas, thus creating a complex mixture of amorphous and crystalline phases) or fully crystalline – based on the physical structure of the polymer networks.
- Temperature response (positive, negative or thermo-reversible), pH response (cationic, anionic), electric signal-sensitive, light, sound or magnetic field sensitive – according to the environment stimuli responses;
- According to preparation methods – graft polymerization, cross-linking polymerization, radiation cross-linking;

1.4.3 Hydrogel Preparation

Hydrogels can be created by cross-linking homopolymers or copolymers, resorting to physical or chemical paths in order to build a three-dimensional structure with specific chemical and mechanical properties. The cross-linking

reaction can be made by covalent (chemical hydrogel) or non-covalent interactions (physical hydrogel), and the network structure comprises the formation of branched polymers in a primary stage, which tends to increase in size and outcome in a gel/network structure. [21][22]

Physical cross-linking results in hydrogels whose networks are held together by secondary non-covalent forces, such as ionic or hydrophobic interactions as well as hydrogen bonds. This kind of synthesis does not require the use of a cross-linking agent and can be attained by different techniques like heating or cooling of a polymer solution, ionic interactions, hydrogen bonding, and heat-induced aggregation, among others. [21][22]

Chemical cross-linking results in permanently linked covalent bonds. Like mentioned before, the swelling equilibrium of this hydrogels depends on the cross-linking density and also on the intensity of the interaction between the polymer and water. This kind of cross-linking can be obtained by polymerization which takes advantage of the functional groups present on the polymer backbone; by polymerization in the presence of a cross-linking agent; and by a reaction of polymer-polymer cross-linking as well. [21][22]

In this work, the main goal is to achieve chemical cross-liking of dextran, because the intended hydrogel is synthesized in the presence of a cross-linking agent, which involves active reaction sites like the hydroxyl groups in the polysaccharide backbone.

1.5 Hydrogels In Drug Delivery Systems

As mentioned before, hydrogels are polymers with a three-dimensional matrix and a semisolid morphology that can absorb a large volume of water. This occurs because they comprise ionizable functional groups (hydrophilic) which affect their permeability, mechanical stability, and chemical structure. The porosity can

be controlled by tuning the density of cross-links in the gel matrix and the affinity to the aqueous medium in which they are swollen. This high porosity allows the loading of drugs into the matrix and subsequent release while interacting with an aqueous solution. The water causes the polymer to swell and consequently release the drug at a certain rate, dependent on the diffusion coefficient of the drug molecules through the gel network. [23]

A major benefit of using hydrogels to develop drug delivery systems is mostly related to pharmacokinetics, since they have a formulation in which the drug slowly elutes and maintains a high local concentration in the surrounding tissues over an extended period of time. Besides, the degradation products must have biocompatible properties and should not show toxic, allergic or inflammatory effects. [23]

Biodegradable polymeric systems for controlled release have been widely studied in biomedical applications since they can prevent the recourse to an invasive procedure, such as surgery, after treatment. [24]

Aside from this favourable features, drug delivery systems based on hydrogels have not yet found a suitable way into clinical applications. This occurrence is mainly due to the quantity and homogeneity of the loaded drug and the fairly prompt drug release over the course of a few hours or days, which act as diminishing factors to its efficacy and may cause harmful side effects. [25]

Amongst the several polymers employed to form hydrogels, polysaccharides show numerous advantages when compared to other macromolecules. As mentioned before, they are abundant and easily available from renewable sources such as algae, plants, cultures of microbial strains and through recombinant DNA techniques. In addition, they comprise structures and properties not easily mimicked in laboratory and their relatively ease of production makes most polysaccharides cheaper than synthetic polymers. [26]

Due to their high water content, soft and rubbery consistency and low interfacial tension with water or biological fluids, polysaccharide-based hydrogels resemble natural soft tissue much more than other types of polymeric biomaterials. They partake a physico-chemical resemblance to the native extracellular matrix, both in composition and mechanical properties, making them significantly biocompatible and permeable to oxygen, nutrients and other metabolites. Likewise, they are fairly deformable and can be produced with a wide range of physical forms like macroscopic networks such as films, and smaller networks such as micro- or nanoparticles. Alongside these favourable features, polysaccharide-based hydrogels have been present in clinical practice and experimental medicine for a wide range of applications, specifically in tissue engineering, cellular immobilization, diagnostics and drug delivery and retain their properties well documented. [23]

1.5.1 Diffusion Process

When the matrix of a drug delivery hydrogel comes in contact with surrounding water or other biofluids, a drug concentration gradient will arise between the molecules within the hydrogel and the surrounding fluid. This gradient will force the movement of the drug from the high concentration site to the surrounding fluid, at a lower concentration, as represented in Figure 1.7.

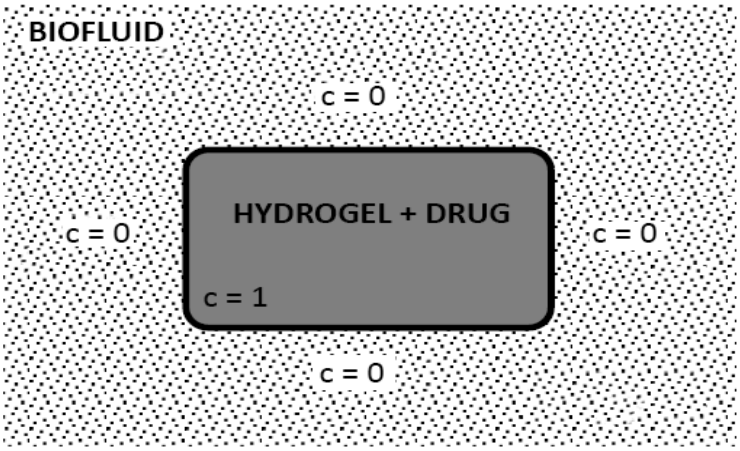

Figure 1.7 – Hydrogel with an encapsulated drug, immersed in a biofluid, with differences in concentration.

This occurrence is best described by Fick's laws of diffusion or Maxwell-Stefan equations. [27][28]

When there is no movement of the hydrogel boundary (hydrogel is not swelling), the diffusion is known as Static Drug Delivery. In contrast, when the hydrogel is undergoing a swelling process, it is known as Dynamic Drug Delivery. [27]

In theory, the diffusion of a solute is not possible within the matrix when the pore sizes are similar to the size of the solute molecule, as represented Figure 1.8, where a protein is entrapped inside the pore and there is no way of diffusion. As explained before, the mesh sizes are affected by several factors such as the degree of crosslinking of the hydrogel, chemical structure of the constituting monomers, and other external stimuli (temperature, pH and ionic strength). [28]

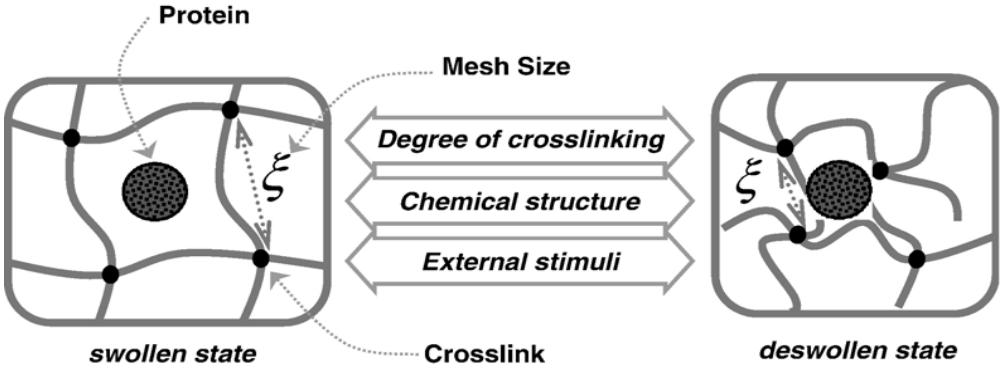

Figure 1.8 – Mesh sizes and influencing factors in drug delivery. [28]

1.5.2 Drug Releasing Mechanisms

Hydrogel based drug releasing mechanisms can be classified as: diffusion-controlled, swelling-controlled, chemically-controlled, and environmentally responsive systems. [23]

Diffusion-controlled release systems can be classified either as reservoir or matrix systems. In both of the devices the release of the drug takes place by diffusion through the mesh. Moreover, hydrogel systems diffusivities of

encapsulated molecules depend on the degree of swelling, cross-linking density and mesh sizes within the matrix of the hydrogels. [27][28]

In diffusion reservoir systems, a drug depot in the shape of a capsule or sphere is encircled by a polymeric hydrogel membrane, as exemplified in Figure 1.9. By restraining the drug to the center of the device, the diffusion primarily occurs through hydrogel and afterwards to the surrounding environment. [27][28]

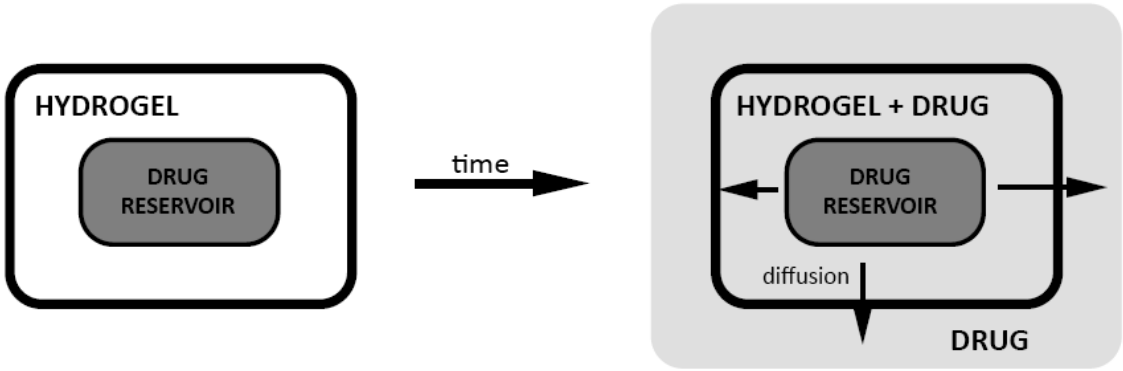

Figure 1.9 – Schematic of a diffusion-controlled reservoir drug releasing mechanism.

In diffusion matrix systems, the drug is distributed throughout the three-dimensional structure of the hydrogel, as shown in Figure 1.10. This is accomplished by mixing the drug with the polymer powder and subsequently compressing the mixture. The drug release takes place by diffusion when the mesh pores are filled with water, and this is dependent on the mesh sizes within the matrix. [27][28]

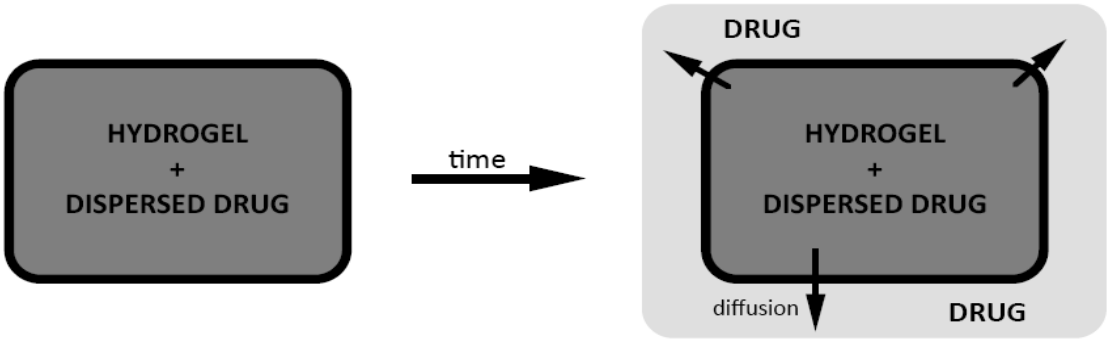

Figure 1.10 – Schematic of a diffusion matrix drug releasing system.

In swelling-controlled release systems, the drug is dispersed throughout the network of the polymer, as in a matrix device, illustrated in Figure 1.11. [27][28]

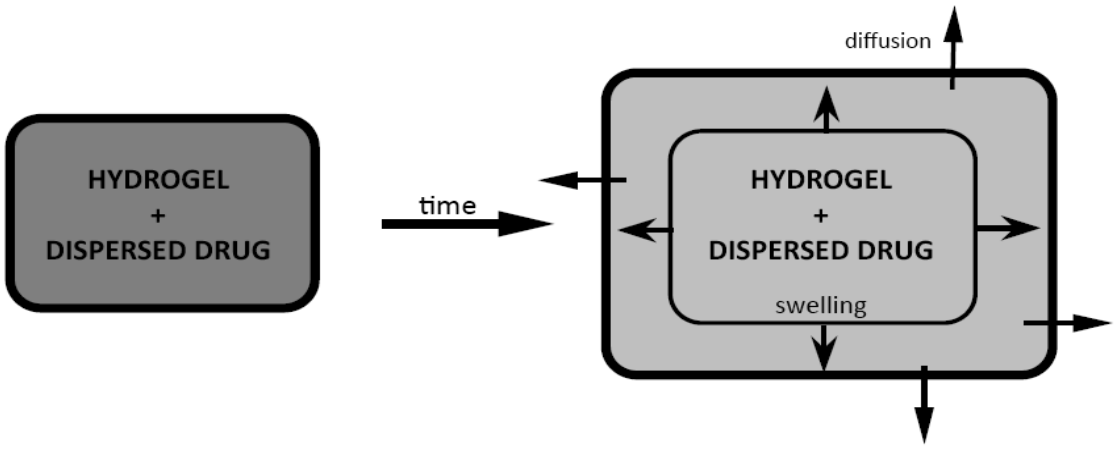

Figure 1.11 – Schematic of a swelling-controlled drug releasing system

When dried, the hydrogel stands in a glassy state, but once it begins to interact with water or other biofluids, it starts to swell and its boundaries expand in size, as showed in Figure 1.12. While the swelling occurs, the glass transition temperature of the polymer decreases, allowing a relaxation between the molecular chains and establishing a more rubbery consistency, hence permitting the diffusion of the drug outside of the swollen area. [27][28]

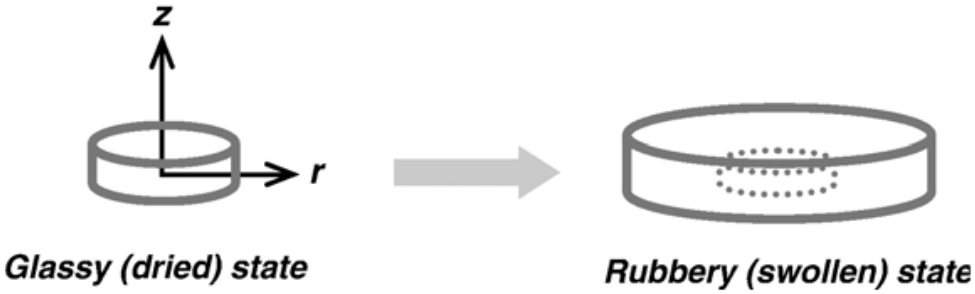

Figure 1.12 – Illustration of the change in size of a hydrogel in a dry and swollen state. [28]

Fundamentally, in swelling-controlled delivery systems, the rate of the drug release is dependent on the swelling rate of the hydrogel network, while in diffusion-controlled systems, the Fickian diffusion dictates the molecule release progression. In some cases, a combination of a swelling-controlled and diffusion-controlled mechanisms is employed. [27][28]

Chemically-controlled systems are controlled by specific reactions occurring within the hydrogel matrix. This reactions can be cleavage of polymer chains via hydrolytic or enzymatic ways; reversible or irreversible reactions taking place between the hydrogel network and the drug to release; erosion in the surface or the bulk contributes to the rate of release; drug-binding moieties may be incorporated in the hydrogels, being the binding equilibrium responsible for the release rate. [27][28]

1.5.3 Dextran Hydrogels In Drug Delivery

As mentioned before, dextran is a glucose homopolysaccharide with a significant amount of consecutive α-(1,6) linkages in the main chain, from which α-(1,2), α-(1,3) and α-(1,4) branches emerge. It is a widely used polysaccharide in the pharmaceutical field, and a few examples of potential applications of dextran in drug delivery systems found in literature are illustrated below.

There have been several trials on the use of dextran polymers for colon drug delivery. Particularly, Simonsen *et al.* studied the stability of dextran hydrogels in *in vitro* emulations of the human small intestine and colon environments to evaluate if the dextran matrices were appropriate carriers for site specific drug delivery. The hydrogels turned out to be stable in those conditions after a long period of time, which could be shortened or lengthened by changing the structure or the thickness of the hydrogel, confirming that to higher values of cross-linking correspond lower values of mass loss. [29][30]

A promising field in drug delivery systems is based on bioresponsive hydrogels, which can regulate the release through the response to environmental stimuli by swelling or de-swelling. A thermoresponsive and biodegradable dextran-based hydrogel was developed by Huang & Lowe to encapsulate and release hydrophilic drugs in response to a change in temperature. [31]

Zhang *et al.* developed bioresponsive dextran-hydrogels made by preparing pH-sensitive hydrogel membranes for drug delivery and tissue engineering. The hydrogel presented COOH groups in its structure that were stimuli responsive, increasing the porosity of the matrix in response to an upturn of pH and ionic strength. Since the hydrogel swelling was pH dependent, the diffusion of an encapsulated protein increased with pH and ionic strength. This model showed that the membrane had a reversible response as well, allowing to observe the change in density and cross-linking so that an ideal balance relating degradation rate and mechanical properties could be commanded. [32]

1.6 5-Fluorouracil

In the treatment of tumoral cancer the most frequent procedure is surgical removal, however, a complete removal of the tumor is a difficult task to succeed. To overcome this issue, surgery is often followed by irradiation or systemic chemotherapy to eradicate the remaining malignant cells and prevent tumor recurrence. Systemic chemotherapy often is associated with several limitations such as reduced efficiency and precision in the treatment of the targeted tumor cells, severe toxicity, and other undesirable side effects derived from a non-specific distribution of the treatment to healthy neighbouring tissues. [33]

Targeted drug delivery systems, as explained before, are a way to overcome these limitations in cancer treatment due to their site-specific drug delivery and prolonged action periods, reducing systemic drug levels and by this decreasing the incidence of side effects in healthy tissues. [33]

The chosen drug to incorporate in the prepared drug delivery hydrogel is the fluoropyrimidine 5-fluorouracil, which is an antimetabolite drug. Antimetabolite drugs function at the level of preventing crucial biosynthetic

processes or inhibition of the regular functions of macromolecules, such as DNA and RNA, after its incorporation in these systems. [34]

This drug was first synthesized by Heidelberger *et al.* in 1957, after observing that rat hepatomas integrate radiolabelled uracil more eagerly than non-malignant tissues, demonstrating that the enzymatic pathways for uracil and its analogues differed between malignant and normal cells. This fact verified that the uracil metabolism was a potential target for antimetabolite chemotherapy. [35]

5-Fluorouracil differs from uracil by changing a fluorine atom in place of hydrogen at the carbon-5 position of the pyrimidine ring, as shown is Figure 1.13.

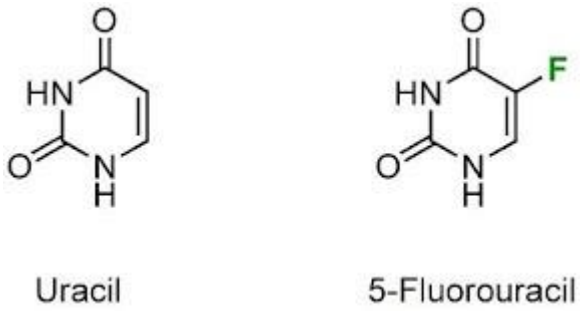

Figure 1.13 – Structure of uracil and 5-fluororacil.

5-Fluorouracil is among the numerous anticancer drugs used in therapy, and stands as a hydrophilic drug extensively used in systemic chemotherapy of solid tumors, such as breast, colorectal, and brain cancer. Due to its structure, 5-Fu interferes with the nucleoside metabolism and might be incorporated into RNA or DNA, leading to cytotoxicity, cell death and preventing cell growth. [33]

Being an analogue of uracil, 5-Fu promptly enters the cell by using the same facilitated transport mechanism as uracil, where it is converted intracellularly into three main active metabolites, illustrated in the metabolic pathways in Figure 1.14 - fluorodeoxyuridine monophosphate (FdUMP), which inhibits enzyme thymidylate synthase (TS), affecting DNA replication and repair; fluorodeoxyuridine triphosphate (FdUTP), which is misincorporated into DNA instead of uracil and leading to cell death; and fluorouridine triphosphate (FUTP),

which is extensively incorporated into RNA, disrupting its normal processing and function and eventually leading to cell death. Up to 80% of administrated 5-Fu is mainly catabolized in the liver by dihydropyrimidine dehydrogenase (DPD), where it is amply expressed. [34][36]

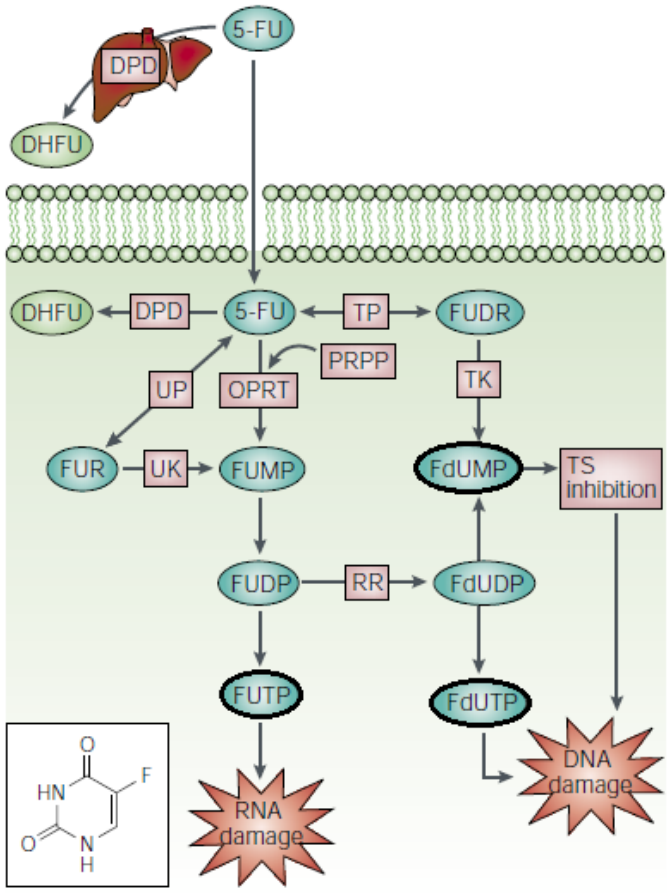

Figure 1.14 – Metabolic pathways of 5-Fu. [34]

When administrated, 5-Fu has several pharmacokinetic limitations, including poor maximum drug concentration, a short plasma half-life of 10 to 20 minutes if administrated intravenously, and, if administrated orally, poor absorption and quick disposability. Furthermore, a severe increase of 5-Fu concentrations in plasma shows harsh side effects and the antitumor effects depend on the duration of the exposure rather than the concentration levels. With this, it is appropriate to say that 5-Fu has a time-dependent method of action rather than dose-dependent. [33][37]

These disadvantages make 5-Fu a suitable candidate for encapsulation in hydrogels, in order to maintain a prolonged and controlled drug delivery and improve the stability and bioavailability of the drug in the specific sites.

1.7 Characterization Techniques

1.7.1 Chemical Characterization

- **Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR FTIR)**

In order to get detailed information about the prepared samples on a structural level, each compound was submitted to ATR FTIR analysis. Since polysaccharides have the particularity of having glycosidic bonds, it is possible to analyse their glycosidic bridge vibrations by spectroscopy.

Infrared spectroscopy is the study of the interaction of infrared light with matter. A beam of infrared light is targeted at the desired sample and the absorbed wavelengths depend on the molecular vibrations in the compound. With this, based on the infrared absorption, both chemical and structural information can be assessed. In short, this technique centers on a measurement of absorbance peaks at certain wavenumbers (expressed in cm^{-1}) of IR radiation by the sample when its structural units vibrate, allowing the identification of the structural groups present in the sample, since each group denotes different absorbance peaks. [38][39]

ATR FTIR spectroscopy consists of aiming infrared light to an interface located between an infrared transparent material with a high refractive index ATR crystal (internal reflection element – IRE) and a sample of the compound to be studied placed on the surface of the IRE. This is fundamentally a surface layer technique in which the evanescent wave only interacts with the portion of the sample in direct contact with the ATR surface, making the whole procedure very

quick and direct, non-destructive and only requiring small amounts of dried material. [39] [40]

The exit signal of FTIR spectroscopy is a spectrum, which presents the wavenumber (cm^{-1}) in the horizontal axis and the transmittance (%) in the vertical axis. [39]

1.7.2 Drug Releasing Quantification

- **Ultraviolet/Visible Spectrophotometry**

Spectrophotometry studies how a sample responds to light. Essentially, a beam of light goes through a substance or solution and some of that light may be absorbed, while the remaining is transmitted through the sample. The ratio between the light entering the sample (I_0) and the light exiting (I_t) is defined as transmittance (T) as follows in Equation 1.1. This relates to absorbance as the negative logarithm of transmittance, as shown in Equation 1.2. The process is illustrated in Figure 1.15.

$$T = \frac{I_T}{I_0} \quad (1.1)$$

$$A = -\log T = -log\frac{I_T}{I_0} \leftrightarrow A = log\frac{I_0}{I_T} \quad (1.2)$$

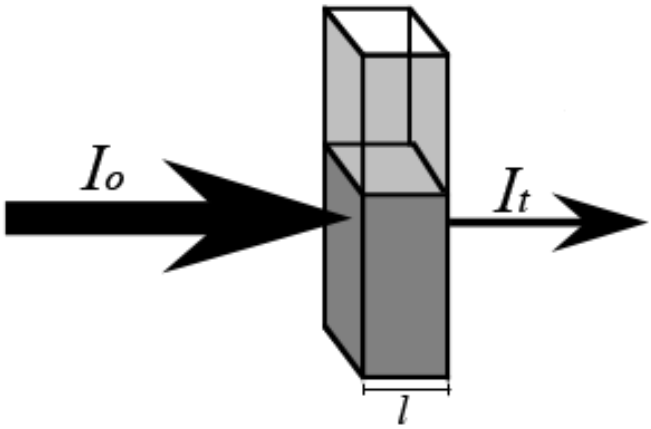

Figure 1.15 – Illustration of the absorbance process in a spectrophotometer cell.

An important principle to analyse the absorption is the Beer-Lambert Law presented in Equation 1.3, stating that for an ideal solution there is a liner relationship between concentration (c) and absorbance (A) as long as the path length (l) is kept constant. The absorptivity of the substance ($\boldsymbol{\varepsilon}$) and the length of the path are constant for the same set of experiments, which produce a straight line when plotting the absorbance against the concentration.

$$A = \varepsilon c l \tag{1.3}$$

Typically, a calibration curve is prepared by plotting a series of concentrations with the respective absorptions, which allows to know the concentration of an unknown sample by measuring its absorbance. [41][42]

In this study, UV-Vis spectrophotometry was the recurring method to determine the amount of released drug by the prepared samples. A beam with a fixed wavelength of 265 nm in the spectrophotometer was targeted through two cuvettes, one with a reference liquid and the other with the drug diluted in that same liquid. The measured value stands for the difference in absorbance between the two cuvettes, allowing to know the concentration of the drug by recurring to the calibration curve prepared beforehand.

2 Materials and Experimental Methodology

2.1 Materials

The chosen polymer to prepare the hydrogels was dextran, produced by *Leuconostoc*, with a molecular weight of 450.000 to 650.000 Da. In an early stage of the work to run initial tests to establish the procedure, dextran with a molecular weight of 70.000 Da was used. Both polymers were acquired at Sigma-Aldrich.

The resorted organic solvent was dimethyl sulfoxide (DMSO) at 99%, acquired in Arcos Organics, previously dried with calcium hydride and distilled with the appropriate apparatus. Minor additional tests were driven with two other solvents - tetramethylene sulfone (sulfolane) at 99% and propylene carbonate at 99%, both acquired at Sigma-Aldrich. These tests were not successful, since dextran did not dissolve in the referred solvents.

The main cross-linking agent cannot be mentioned due to terms of confidentiality and it will be referred as cross-linking agent X hereafter. Several other compounds were employed as additional cross-linking agents, being the main two: 1,2,3,4-Butanetetracarboxylic acid (BTCA) at 99% with a molecular weight of 234.16 g/mol and 2,2-bis-(hydroxymethyl)-propionic acid (Bis-MPA) at 98%

with a molecular weight of 134.14 g/mol, both from Sigma-Aldrich. Other compounds were tested as well as additional cross-linking agents, for instance, sebacic acid at 98% from Tokyo Chemical Industries with a molecular weight of 202.25 g/mol; fumaric acid at 99% from Tokyo Chemical Industries with a molecular weight of 116.07 g/mol; adipic acid at 99.6% from Sigma-Aldrich with a molecular weight of 146.14 g/mol; succinic acid at 99% from Acros Organics with molecular 118.09 g/mol; and poly(vinyl alcohol) at 98% from Sigma-Aldrich with a molecular weight of 13.000 to 23.000 g/mol.

The precipitation of the gels was performed in 2-propanol (isopropyl alcohol) acquired in Fischer Scientific and Chem-lab.

The drug for encapsulation was 5-fluorouracil (5-Fu) at 99% with a molecular weight of 130.08 g/mol, acquired at Sigma-Aldrich.

The swelling trials of the prepared films and the releasing profile of the drug were performed in a medium with a standard clinical saline solution of sodium chloride (NaCl) at 9 mg/ml (0.9%) from Labesfal.

2.2 Equipment

The FTIR analysis to evaluate the chemical structure of the prepared compounds was driven using a spectrometer JASCO FTIR-4200 with a wavelength comprised between 550 and 4000 cm^{-1}. The resolution of the spectrometer was 4 cm^{-1} and it was engaged in ATR mode to acquire the spectra. The theoretical principles of FTIR analysis were explained previously in Chapter 1.

In order to measure the amount of released drug from the prepared compounds, UV-Vis spectrophotometry was the resorted technique, using a spectrophotometer JASCO V-530 with tungsten-halogen and deuterium lamps, enforcing a fixed wavelength of 265 nm. Absorbance was the unit in measurement,

and through the calibration curve of the drug, prepared beforehand, the concentration of the drug in the solutions was obtained.

Certain samples were submitted to Scanning Electron Microscopy to analyse some surface properties, and the equipment was a TESCAN VEGA3-SEM Analytical Scanning Electron Microscope.

2.3 Methodology

The majority of the procedures were performed with basis on previous works by the polymer research group of the Department of Chemical Engineering of the University of Coimbra and the main goal was to improve the cross-linking properties of the prepared samples and prolong the swelling process with a slower swelling profile.

In a preliminary phase of the studies, a test sample was prepared in order to establish the basics of the procedure. For this, dextran with a molecular weight of 70.000 Da was the used polymer. Later on, all of the synthesis were executed using dextran with a molecular weight of 450.000-650.000 Da, by decision of the group. The chosen organic solvent in all the discussed synthesis was DMSO, previously dried and distilled, with exception in two circumstances, where attempts to dissolve dextran in sulfolane and other in propylene carbonate were not carried out successfully. Additionally, the selected cross-linking agent and the used quantities are not mentioned due to terms of confidentiality, and it will be referred hereafter as cross-linking agent X (CL-X).

Essentially, three slightly different types of hydrogels synthesis were carried on - Type I, Type II and Type III synthesis.

(**NOTE**: The distinction among the synthesis was cleared out by bearing in mind the different involved compounds, with every other aspect kept unchanged. In

short: Type I synthesis only included the polymer dextran plus the cross-linking agent X; Type II synthesis covered tests by adding different compounds (mostly dicarboxylic acids) to dextran plus the cross-linking agent X; Type III synthesis was analogous to Type II by adding other compounds to dextran plus the cross-linking agent X, however the prepared hydrogels by this method had the most promising features and therefore the chosen to proceed until the end of the studies, hence this distinction.)

2.3.1 Hydrogel Preparation

Type I Synthesis

Type I synthesis was executed primarily by adding DMSO in a round-bottom flask and rising its temperature to 40ºC in a water bath. This was followed by addition of dextran until the polymer dissolved completely. After dissolution, the flask was degasified with a flow of nitrogen (inert gas) for ten minutes. After this step, the cross-linking agent X was added and the reaction was left overnight at a temperature of 40ºC for the cross-linking to occur completely and to obtain a final product in the form of a gel-like solid.

The details and quantities of the prepared samples by Type I synthesis are presented in Table 2.1.

The majority of the synthesis were a repetition of the same procedure, focusing on improving the methodology. The only exception was on sample 3.A, where the amount of cross-linking agent X was increased by 25%. All of the synthesis by this method were able to form gels.

Table 2.1 – Details of the prepared samples by Type I synthesis.

Sample	Polymer type	Polymer mass (mg)	DMSO volume (ml)	CL-X mass (mg)	Gel formation
1.A	Dextran (mw = 70.000 Da)	500	10	x	yes
2.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes
3.A	Dextran (mw = 450.000-650.000 Da)	500	10	1.25x	yes
4.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes
5.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes
6.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes
7.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes
8.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes
9.A	Dextran (mw = 450.000-650.000 Da)	500	10	x	yes

Type II Synthesis

Type II synthesis followed the same basic steps as Type I, with exception in the addition of a supplementary compound. As precedently, DMSO was added into a flask and the temperature risen to 40ºC, followed by addition of dextran plus a secondary compound and left until the dissolution occurred completely. In detail, the employed secondary compounds were succinic acid, fumaric acid, sebacic acid, adipic acid (all dicarboxylic acids) and polyvinyl alcohol (PVA). The procedure was carried on according to the previous synthesis, by degasifying the flask with nitrogen and posteriorly adding the cross-linking agent X, leaving the reaction running overnight at 40ºC in order to obtain a final product in the form of a gel-like solid.

Once again, the details of the prepared compounds by Type II synthesis are displayed in Table 2.2.

All the synthesis were carried using the same amount of dextran (450.000-650.000 Da), DMSO and cross-linking agent X. The additional compounds had different quantities in order to test the final properties of the hydrogels. Nearly all products were able to form gel-like solids, with exceptions for fumaric acid, where the reaction turned black and was discarded, and PVA, where the final product was not quite a gel but instead a liquid-gel mixture, showing that the cross-linking did not occur properly.

Table 2.2 – Details of the prepared samples by Type II synthesis.

Sample	Polymer mass (mg)	DMSO volume (ml)	Additional compound	Additional compound mass (mg)	CL-X mass (mg)	Gel formation
1.B	500	10	Succinic Acid	225	x	yes
2.B	500	10	Fumaric Acid	250	x	no
3.B	500	10	Succinic Acid	100	x	yes
4.B	500	10	Succinic Acid	100	x	yes
5.B	500	10	Sebacic Acid	100	x	yes
6.B	500	10	Succinic Acid	100	x	yes
7.B	500	10	Sebacic Acid	55	x	yes
8.B	500	10	Adipic Acid	55	x	yes
9.B	500	10	PVA	100	x	no

Type III Synthesis

Type III synthesis followed exactly the same procedures as Type II synthesis. The difference, is in the fact that the prepared compounds displayed better properties and were the chosen to carry on the subsequent studies. In a personal opinion, I assumed it was appropriate to separate these compounds from the others, since they were target for more developed studies, including several swelling studies and the drug encapsulation. As described, a flask with DMSO was heated to 40ºC, followed by addition of dextran the additional compound and left to dissolve for about one hour. After this step, degasification was performed with nitrogen for ten minutes and subsequent addition of the cross-linking agent X. The reaction was left overnight at 40ºC in order to obtain a product in the form of a gel-like solid. The additional compounds were 1,2,3,4-Butanetetracarboxylic acid (BTCA) and 2,2-bis-(hydroxymethyl)-propionic acid (Bis-MPA).

The first tests comprised different amounts of BTCA, in order to determine which amount would be more suitable for film formation. Several samples had the exact same characteristics due to the fact that for further stages more compound was necessary to form films for the swelling tests and drug encapsulation.

Details of the Type III synthesis are displayed on Table 2.3.

Table 2.3 – Details of the prepared samples by Type III synthesis.

Sample	Polymer Mass (mg)	DMSO volume (ml)	Additional compound	Additional compound mass (mg)	CL-X mass (mg)	Gel formation
1.C	500	10	BTCA	50	x	yes
2.C	500	10	BTCA	25	x	yes
3.C	500	10	BTCA	50	x	yes
4.C	500	10	BTCA	100	x	yes
5.C	500	10	Bis-MPA	50	x	yes
6.C	1000	20	BTCA	50	x	yes
7.C	1000	20	BTCA	75	x	yes
8.C	500	10	BTCA	30	x	yes
9.C	500	10	Bis-MPA	50	x	yes
10.C	500	10	BTCA	30	x	yes
11.C	500	10	Bis-MPA	50	x	yes
12.C	500	10	BTCA	30	x	yes
13.C	500	10	Bis-MPA	50	x	yes
14.C	500	10	BTCA	30	x	yes
15.C	500	10	Bis-MPA	50	x	yes
16.C	500	10	BTCA	30	x	yes
17.C	500	10	Bis-MPA	50	x	yes

All of the samples resulting from Type I, Type II and Type III synthesis will be discussed ahead on Chapter 3 for a comprehensive analysis regarding their properties and engaged tests.

2.3.2 Gel Precipitation In 2-Propanol

Following the synthesis procedure, all the prepared samples able to form a gel-like material were washed in 2-propanol to ensure the removal of the solvent (DMSO). The gels originated from the synthesis were disaggregated with aid of a spatula and transferred to a flask to be filled with 2-propanol. The washings were carried for the duration of 4 hours, with full replacement of 2-propanol in the meantime, after approximately 2 hours, in order to guarantee a complete elimination of the DMSO. After completion of this step, the resulting precipitate would be deposited on the bottom of the flask. To remove the exceeding 2-propanol, a filtering system with a vacuum pump connected to a vented Erlenmeyer with a glass filter funnel on top was set, where the 2-propanol with the precipitate was poured. The 2-propanol would flow into the Erlenmeyer and the solid sample would stay on the filter.

To assuredly guarantee a complete removal of 2-propanol, the solid samples were transferred into a round bottom flask and placed in a rotary evaporator for one hour and later stored overnight enclosed into a vacuum desiccator.

This step showed up to be crucial to acquire the necessary properties within the compounds aimed for the film formation, and it will be discussed ahead on Chapter 3 in section 3.1.2.

2.3.3 Film Formation

After obtaining the dried solid samples, the compounds were disaggregated with help of a mortar and a pestle in the interest of creating a thin powder. Subsequently, 100 milligrams of the powder were measured and submitted to a press to form a film.

After film formation, swelling studies were performed in order to study the swelling and degradability properties of the compounds. To this end, the films were

submerged into 8 ml of a saline solution (0.9 %) and placed inside an incubator at 37°C. The mass variation (water intake) over the days was the variable under study.

The compounds which were able to form films and their respective swelling tests will be discussed further on Chapter 3.

2.3.4 Drug Encapsulation

Once the desired properties were obtained in the prepared compounds and film formation tests, the final step of the work concerned the encapsulation of the drug 5-Fu into the polymer films. Two methods for encapsulation were implemented, both using 6 mg of 5-Fu for 100 mg of polymer powder. The samples handled for this phase of the study were entirely result of Type III synthesis, specifically 8.C, 9.C, 12.C, 13.C, 16.C and 17.C.

Encapsulation method I was accomplished by mixing 6 mg of 5-Fu with 100 mg of the desired polymer compound, previously disaggregated with aid of mortar and pestle to form a thin powder. Afterwards, the mixture would be submitted to a press to achieve the film formation. This method is essentially similar to the presented in 2.3.3., with the exception being in the inclusion of the 5-Fu in the powder mixture.

The encapsulation method II was equivalent to encapsulation method I, however had one extra step: after preparation of the films, they were immersed into a polylactic acid (PLA) and polyester (50/50) $CHCl_3$ (chloroform) solution, aiming to create a coating.

To monitor the drug release, the films were submerged into 8 ml of saline solution and placed inside an incubator at 37°C, similarly to the swelling studies. In order to quantify the amount of drug release over the days, a spectrophotometer was the resorted equipment to measure the absorbance of the drug dissolved

through the saline solution, and with the help of the calibration curve (presented in section 2.3.5.), to know the concentration and consequently the amount released.

The drug releasing results will be subjected to further discussion on Chapter 3 in section 3.2.2.

2.3.5 5-Fu Calibration Curve

Concerning the relation between the concentration in solution of the drug 5-Fu and the absorbance measured in the spectrophotometer, a calibration curve was established. By resorting to this curve, we are afterwards able to determine the amount of released drug by the prepared samples to the saline solution.

In favour of preparing different 5-Fu concentrations in several solutions, 20 milligrams of 5-Fu were dissolute in saline solution inside a 25 millilitre dissolution flask, thus providing a concentration of 0.8 mg/ml. Subsequently, with the obtained concentration of 0.8 mg/ml, different volumes of this solution were distributed with a micropipette over seven dissolution flasks with a volume 10 millilitres, therefore obtaining seven different concentrations. In Table 2.4 are presented the details of the dissolutions and the respective measured absorbances by the spectrophotometer.

A full wavelength scan was ran to determine in which wavelength the drug would have its peak of absorbance, being this value at 265 nm. As a result, all measurements performed afterwards were set in a fixed wavelength of 265 nm.

Table 2.4 – Details of the prepared dissolutions to build the 5-Fu calibration curve.

Flask	5-Fu Mass (mg)	Initial Volume (ml)	Final Volume (ml)	Concentration (mg/ml)	Abs
Main	20	25,00	25	0,800	-
1	-	0,05	10	0,004	0,2671
2	-	0,10	10	0,008	0,4580
3	-	0,15	10	0,012	0,6729
4	-	0,20	10	0,016	0,8952
5	-	0,30	10	0,024	1,3019
6	-	0,40	10	0,032	1,7718
7	-	0,50	10	0,040	2,1778

With the known concentrations and respective absorbance, it is possible to draw a graph relating both values, presented in Figure 2.1. The relation obtained from the plot is displayed in Equation 2.1, with a R^2 equal to 0.999, and where y is absorbance and x the concentration, and follows as:

$$y = 54{,}942x \quad (2.1)$$

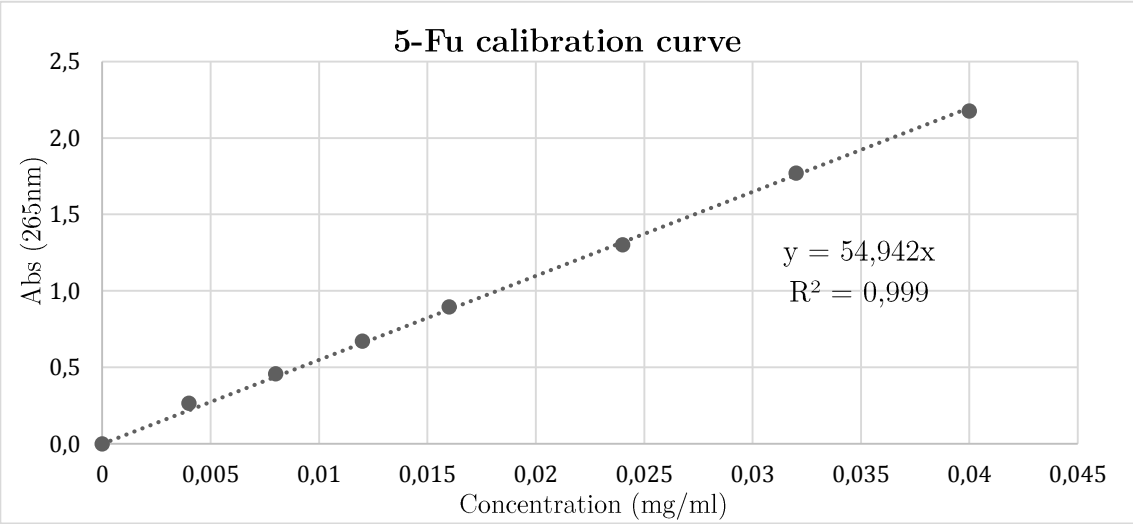

Figure 2.1 – Calibration curve of 5-Fu, relating the measured absorbance and concentration of the solutions.

3 Results and Discussion

3.1 Synthesis

As formerly mentioned in Chapter 2, three types of synthesis for chemical modification of dextran were performed:

Type I synthesis lead to the dissolution of the polymer dextran in DMSO and subsequent addition of the cross-linking agent X. This synthesis is mostly regarded by the reaction between the hydroxyl groups of the dextran chain and the functional groups of the cross-linking agent X, thus producing a cross-linked network in the form of a gel. The reaction is illustrated in Figure 3.1, and stands just as an example of the possible bonding sites, being the functional groups of the cross-linking agent able to bond in any of the three available hydroxyl groups in each dextran molecule within the chain.

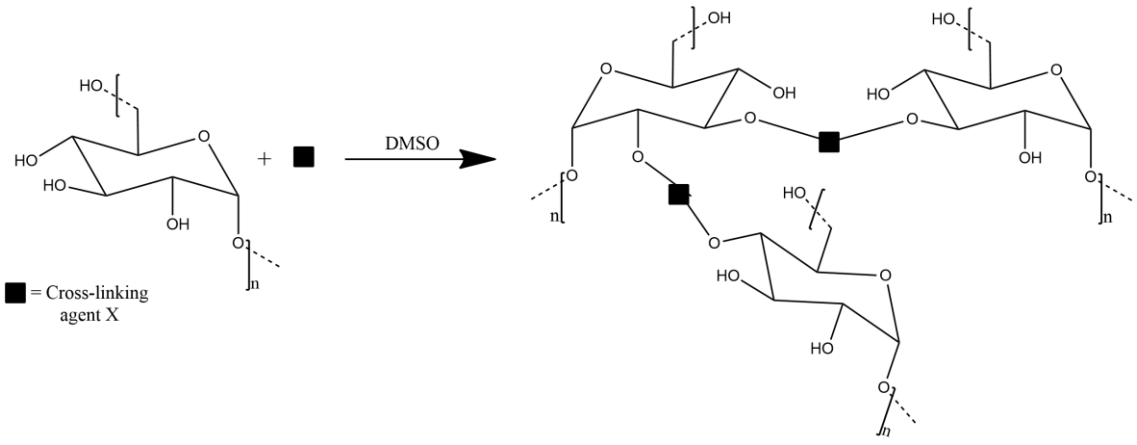

Figure 3.1 – Type I Synthesis reaction between dextran and the cross-linking agent X.

Concerning Type II synthesis, dextran and the additional compound were dissolute in DMSO simultaneously, and subsequently there was addition of the cross-linking agent X. The additional compounds were succinic acid, fumaric acid, sebacic acid, adipic acid (all dicarboxylic acids) and PVA. To illustrate the reaction between dextran, the dicarboxylic acid and the cross-linking agent, is given the example of a reaction with succinic acid as additional compound in Figure 3.2.

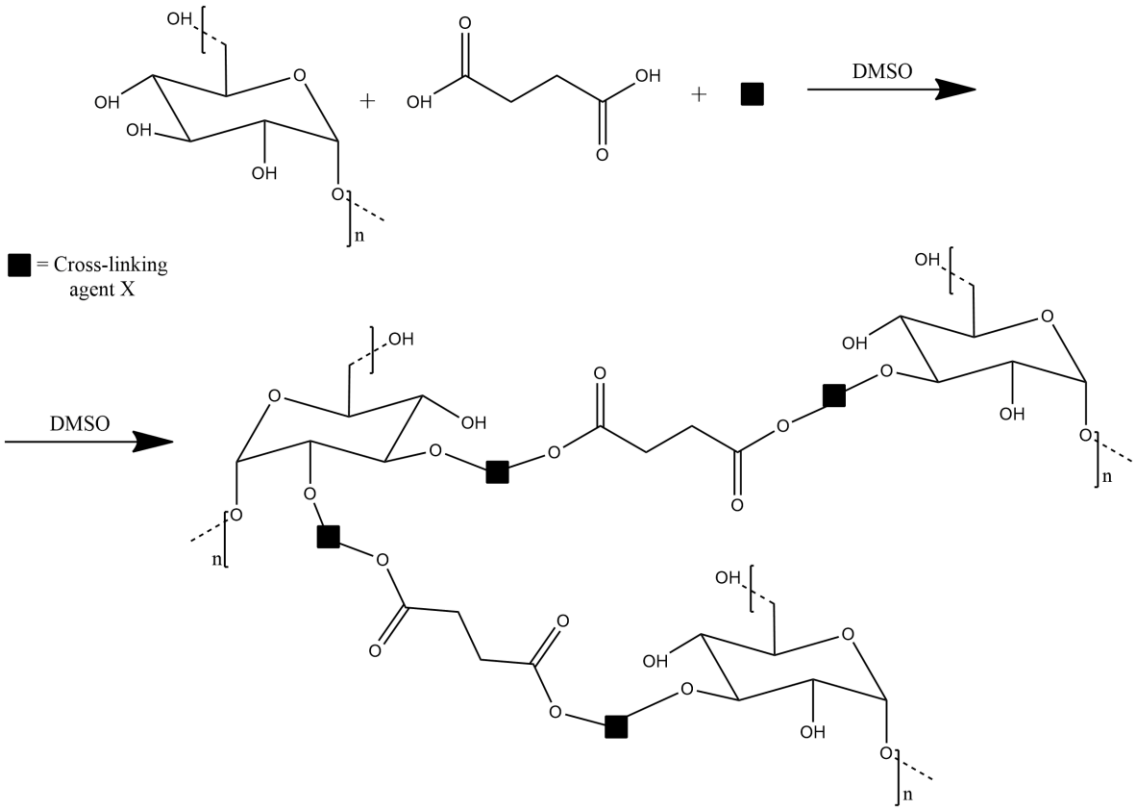

Figure 3.2 – Type II Synthesis reaction between dextran, the cross-linking agent X and succinic acid.

Once again, this illustration is just an example of the possible bonding sites, since the cross-linking could occur in several points of the molecule. Each OH group is a potential site for cross-linking. For instance, the functional groups of the cross-linking agent X could bond two dextran molecules directly through their free hydroxyl groups, or as well bond the dextran molecules to the carboxyl groups present in succinic acid. The bonding may also occur in any of the three free hydroxyl groups present in dextran. With all other dicarboxylic acids the reaction

occurs in the same conditions, owing to the presence of identical carboxyl functional groups.

Type III synthesis comprised the same steps as Type II, as mentioned before. Dextran and an additional compound were dissolved in DMSO simultaneously and afterwards there was addition of the cross-linking agent X. The additional compounds were BTCA and Bis-MPA.

In Figure 3.3 it is visible the reaction between dextran, BTCA and the cross-linking agent in the DMSO medium. Since BTCA is a tetracarboxylic acid, it comprises two more carboxyl groups (4, in total) than the dicarboxylic acids employed in Type II synthesis, therefore more bonding sites are available within the molecule. With this properties, BTCA was introduced in the synthesis with the aim to produce a more cross-linked hydrogel.

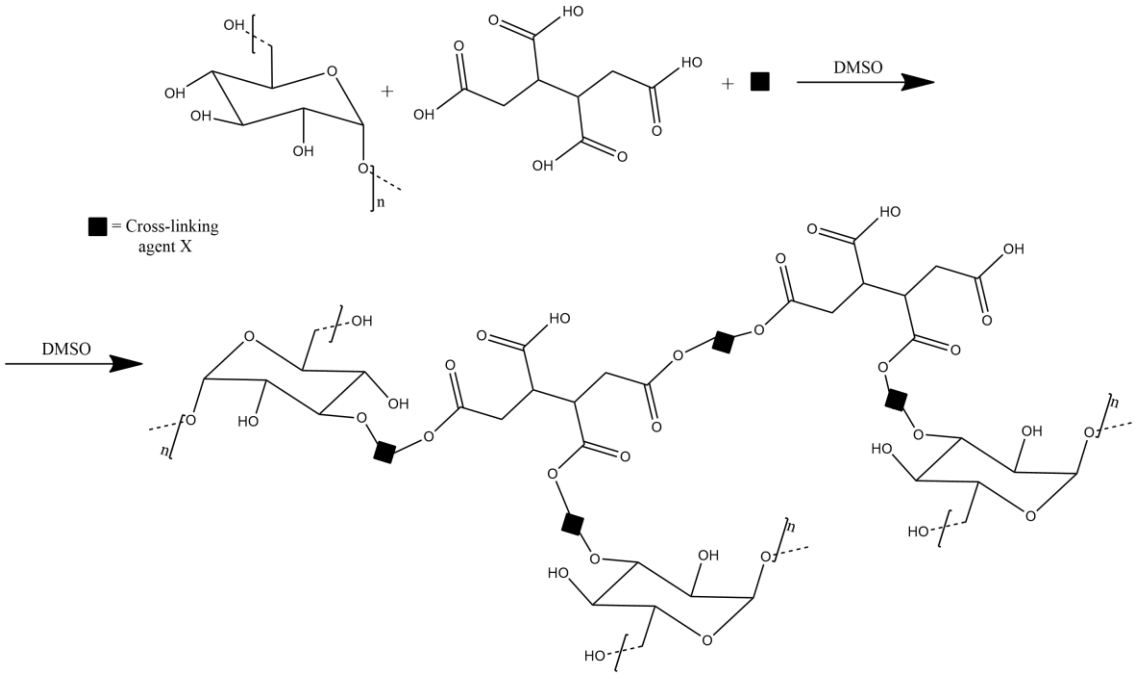

Figure 3.3 – Type III Synthesis reaction between dextran, the cross-linking agent X and BTCA.

The illustrated reaction is just an example of some of the several binding sites, since numerous combinations are possible. The functional groups in the cross-linking agent may bond two dextran molecules directly through its hydroxyl groups,

or bond a dextran molecule to one of the carboxyl groups present in BTCA. All the free hydroxyl and carboxyl functional groups might present a bonding site, therefore, an extremely cross-linked network is able to arise.

In Figure 3.4 is displayed a reaction where dextran and Bis-MPA were dissolved in DMSO and there was subsequent addition of the cross-linking agent X. Bis-MPA comprises two hydroxyl and one carboxyl group, which react with the hydroxyl groups present on dextran and the functional groups present on cross-linking agent X. Bis-MPA was introduced for the same end as BTCA, since it could improve the cross-linking density of the resulting hydrogel. It has been applied in the creation of hyperbranched dendrimers and dendritic polymers for drug delivery. [43]

Several combinations are once again possible due to the amount of free functional groups in dextran (three), in Bis-MPA (three) and in the cross-linking agent X.

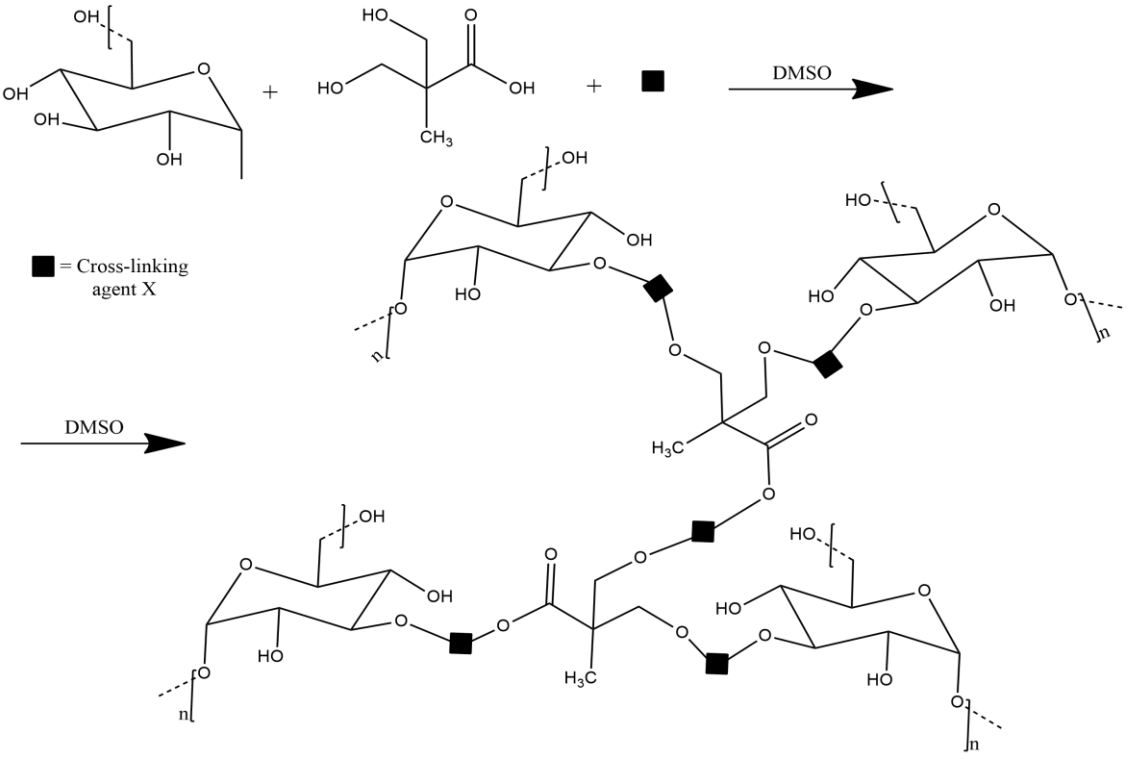

Figure 3.4 - Type III Synthesis reaction between dextran, the cross-linking agent X and Bis-MPA.

Following all the synthesis reactions described in the work, several attempts were made to prepare materials with the necessary properties for further trials, such as film formation and drug encapsulation. A brief summary of all the prepared samples and their results concerning gel formation, dry mass output, film formation and if the film was suitable for testing, follows on Tables 3.1, 3.2 and 3.3, concerning Type I, Type II and Type III synthesis, respectively. Details of the amount of employed compounds in each sample were mentioned in Chapter 2.

Table 3.1 – Type I Synthesis results.

	Sample	Gel formation	Final mass – dry (mg)	Film formation	Film suitable for testing
Type I	1.A	yes	487.5	yes	yes
	2.A	yes	488.2	yes	no
	3.A	yes	1032.1	no	-
	4.A	yes	1131.5	no	-
	5.A	yes	567.6	yes	yes
	6.A	yes	946.5	no	-
	7.A	yes	589.4	yes	yes
	8.A	yes	793.2	no	-
	9.A	yes	695.3	yes	no

Concerning Type I synthesis, the dextran had a molecular weight of 450.000-650.000 Da, with exception of sample 1.A, intended for a synthesis adjustment, with a molecular weight of 70.000 Da. All the samples had the same amount of polymer and cross-linking agent X (with exception in sample 3.A, where the amount of cross-linking agent was raised to 1.25x) and were able to form a gel. After the precipitation in 2-propanol and subsequent drying, the final mass is displayed. A fairly large discrepancy in mass is visible among all samples, and it is also inferred that the film formation was influenced by this inconsistency in mass, since the synthesis procedures were fairly similar among all samples. The majority

of the samples that were not able to form a film had a high mass output, evidencing that perhaps the DMSO was not completely removed from the sample, giving it a rubbery consistency instead of a brittle behaviour. On the other hand, samples with lower mass output were able to form a film suitable for testing, as in case of samples 5.A and 7.A.

Table 3.2 – Type II Synthesis results.

	Sample	Additional compound	Gel formation	Final mass – dry (mg)	Film formation	Film suitable for testing
Type II	1.B	Succinic acid	yes	1103,1	yes	no
	2.B	Fumaric acid	no	-	-	-
	3.B	Succinic acid	yes	1122,2	no	-
	4.B	Succinic acid	yes	810,1	yes	no
	5.B	Sebacic acid	yes	921,8	no	-
	6.B	Succinic acid	yes	926,5	no	-
	7.B	Sebacic acid	yes	763.8	no	-
	8.B	Adipic acid	yes	727,2	yes	no
	9.B	PVA	no	-	-	-

Considering Type II synthesis results, with exception of samples 2.B and 9.B, all samples were able to form a gel. The same occurrence in the final mass output was verified, as in Type I synthesis. In the samples prepared by Type II synthesis, the majority had a high output in mass, due to the rubbery consistency, and only three of this samples were able to form a film, and in none of the cases the film was suitable for further testing.

Type III synthesis was chosen for the majority of the further testing, since it was verified that the samples prepared by this method presented better properties when compared to the previous methods. All of the samples prepared by this type of synthesis were able to form a gel. Samples 8.C through 17.C were a repetition of the same procedures (with BTCA and Bis-MPA), in order to have more compounds available to perform further tests, such as swelling tests and drug encapsulation.

Concerning the final dry mass output, they were significantly lower when compared with the previous methods, and most of the samples were able to form films suitable for testing.

Table 3.3 – Type III Synthesis results.

	Sample	Additional compound	Gel formation	Final mass – dry (mg)	Film formation	Film suitable for testing
Type III	1.C	BTCA	yes	699,0	yes	yes
	2.C	BTCA	yes	633,9	yes	yes
	3.C	BTCA	yes	717,7	yes	yes
	4.C	BTCA	yes	783,6	yes	yes
	5.C	Bis-MPA	yes	585,2	yes	yes
	6.C	BTCA	yes	1533,9	yes	no
	7.C	BTCA	yes	1497,4	yes	no
	8.C	BTCA	yes	633,8	yes	yes
	9.C	Bis-MPA	yes	636,8	yes	yes
	10.C	BTCA	yes	708,6	yes	no
	11.C	Bis-MPA	yes	928,7	yes	no
	12.C	BTCA	yes	661,9	yes	yes
	13.C	Bis-MPA	yes	639,1	yes	yes
	14.C	BTCA	yes	681,5	yes	no
	15.C	Bis-MPA	yes	720,2	yes	no
	16.C	BTCA	yes	510,4	yes	yes
	17.C	Bis-MPA	yes	516,8	yes	yes

3.1.1 Discarded Samples

- **Samples Not Able To Form A Gel**

Considering all the different prepared samples, gel formation was not a drawback, since the majority of samples were able to for gels, as mentioned in section 3.1. The exceptions were in Type II synthesis, where samples 2.B and 9.B were not able to form an appropriate gel. Sample 2.B employed fumaric acid as additional compound. At first, the dextran and the fumaric acid were dissolved in

DMSO, and after few minutes of the addition of the cross-linking agent X, the reaction acquired a dark colouring, being discarded afterwards. Sample 9.B, using PVA as additional compound, comprised all regular steps engaged in other preparations - dissolution of dextran and PVA in DMSO, and subsequent addition of the cross-linking agent X to react overnight, aiming to form a gel. Eventually, the final result had a consistency that resembled a gel, but with a lot of unreacted sample in a liquid state, resulting in a mixture of liquid and a gel phase, being discarded afterwards.

- **Samples Able To Form A Hydrogel But Not Able To Form A Film**

In spite of the majority of the prepared samples were able to form hydrogels, a drawback arose, since several of them did not manage to form films after being dried.

Concerning the nine samples prepared by Type I synthesis, all of them were able to form hydrogels, however, four were not able to form films at all. These samples were 3.A, 4.A, 6.A and 8.A. All the samples were prepared by the same procedure, using 500 mg of dextran and the same amount of cross-linking agent X, with exception for sample 3.A, where 25% more cross-linking agent was employed. All of these samples had a relatively high output in mass and showed a rubbery consistency after being dried.

Concerning the nine samples prepared by Type II synthesis, with exception of the two mentioned in the previous section which were not able to form a gel, all the other seven were able to form gels. From these, four were not able to form films at all, in particular – samples 3.B, 5.B, 6.B and 7.B. Once again, all these samples had a rubbery consistency, demonstrating that probably a poor DMSO removal occurred. Taking the example of samples which had succinic acid added to the

synthesis, an inconsistency is clear among them, since at times a film was achieved, even though if not suitable, and in other cases it was not.

Concerning the seventeen samples prepared by Type III synthesis, all of them were able to form films, even though certain films were not suitable for testing. These circumstances were the sample was able to form a film, yet the film was not suitable for testing will be discussed in the next section.

- **Samples Able To Form A Film, But Not Suitable For Testing**

This section discusses the samples that were, indeed, able to form films after the hydrogel was dried, but the film was not able not perform further tests due to the lack of suitable properties. In particular, after forming films with some of the samples, the films adopted a correct shape, however, not very cohesive and would eventually crumble. In other cases the films were prepared, but after immersion in the aqueous medium for the swelling to occur, the films would disaggregate easily and almost promptly.

As for samples prepared by Type I synthesis, sample 2.A and 9.A were able to form films, however not suitable for testing. In the case of sample 2.A, three films were prepared and immersed into a saline solution to evaluate the swelling behaviour, but they disaggregated immediately after immersion. With sample 9.A, two films were prepared and had the same outcome as in sample 2.A – after immersion in the saline solution they disaggregated immediately.

Concerning the samples prepared by Type II synthesis that were able to form films, but not suitable for testing, we can found three cases: samples 1.B, 4.B and 8.B. With sample 1.B, one film was created and subsequently immersed in a saline solution to evaluate the swelling behaviour, but it disaggregated almost immediately. Regarding samples 4.B and 8.B, one film was prepared with each

sample, but both turned out to have a fragile appearance and behaviour, not being very cohesive and solid.

Concerning the seventeen samples prepared by Type III synthesis, all of them were able to form films, but in six cases those films were not suitable for testing – samples 6.C, 7.C, 10.C, 11.C, 14.C and 15.C. Since the samples prepared by this method were carried on more developed studies and the synthesis was well established, it might be clearer why these particular cases did not form films suitable for testing. In the case of samples 6.C and 7.C, they were prepared using a doubled up formulation, and when dried, they showed a slightly rubbery consistency, when compared to others previously prepared. Two films were created using samples 6.C and 7.C, and showing somewhat fragile and not very cohesive. Furthermore, by observing the mass output, it turned out more than doubled for what would be expected, perhaps showing once more a poor solvent removal.

Samples 10.C and 11.C were prepared at the same time, and the problem might have been slightly different from the mentioned beforehand. Customarily, during Type II and Type III synthesis, the dextran and the additional compound were added at the same time for dissolution in DMSO, however, in these specific cases, there was a delay between the addition of dextran and the subsequent addition of BTCA and Bis-MPA (respectively, in samples 10.C and 11.C). The cross-linking occurred after addition of the agent X, and the process of synthesis was carried by the same methods. After the whole process, the samples seemed somewhat rubbery, and once more, they were not able to be disaggregated properly into a powder. This occurrence might also be attributed to poor DMSO removal in 2-propanol, but since there was this slight difference during the synthesis, it could be a preponderant factor as well, since the bonding between the functional groups of the employed compounds might have resulted in an undesirable way.

Concerning samples 14.C and 15.C, they were able to form films, but the films were once again fairly fragile and not very cohesive, and so, were discarded and not developed into further testing. They had a slightly rubbery consistency and were found very difficult to disaggregate into a thin powder. Once more, this is probably due to poor DMSO removal.

Beyond doubt, the recurrence of samples with a rubbery and not very brittle consistency, and which were not able to form suitable films seemed to be a major issue in the success and reproducibility of the study, as for, this subject is aimed for further clarification in the following section 3.1.2.

3.1.2 Problem in Solvent Removal

Following gel formation, the solvent (DMSO) removal seemed to be a major issue, regarding the desired properties of the compounds in concern to the formation of the films. The majority of the samples that were not able to form films, whereas they were a rigorous repetition of a previous successful procedure or a try-out of a new one, showed a rubbery consistency and a fairly translucent appearance. In order to form appropriate films, the samples had to be disaggregated into a thin solid powder with aid of a mortar and pestle, and for this, they had to retain a brittle and dry consistency. The lack of brittleness might be most certainly attributed to the poor DMSO removal during the precipitate washing with 2-propanol.

Near the end of studies, an enhanced method to obtain a more complete solvent removal was set up – after the gel formation, it was carefully shredded with the purpose of creating smaller portions for a better contact with the 2-propanol phase. When the portions were larger, their inside was not in direct contact with 2-propanol, probably leading to a lack of DMSO removal in those sites. Basically,

the smaller the portions of the gel in contact with the 2-propanol, the higher is the contact surface, leading to better removal of DMSO.

The constant problem compromised the film formation and reproducibility of the procedures, slowing down the process of the study at times, even when the conditions of the synthesis and methods were kept exactly the same. This issue was settled further in the semester than it would be reasonable, leading to misinterpretation of previous results. Perhaps some compounds prepared before were suitable for further testing but since they did not show the desired properties, they were discarded, unaware of this shortcoming. After this procedure, the reproducibility of the experiments was substantially improved.

3.2 Samples Able to Form a Hydrogel and Suitable Films

Swelling tests were performed with seven of the samples which were able to form a hydrogel and a suitable film. These samples were 5.A and 7.A, resulting from Type I synthesis, and samples 1.C, 2.C, 3.C, 4.C and 5.C, resulting from Type III synthesis. The remaining samples that were able to form suitable films were employed in the drug encapsulation phase, particularly, samples 8.C, 9.C, 12.C, 13.C, 16.C and 17.C, all resulting from Type III synthesis. Sample 1.A was able to form a suitable film as well, but it was prepared merely for procedure testing purposes, so it was not target for further studies.

3.2.1 Swelling Tests

This stage intended to evaluate the swelling and degradation properties of the prepared films. The process consisted in submerging the films into 8 ml of a saline solution and store place them inside an incubator at 37ºC to simulate a biological environment. The majority of the prepared films had a dry mass of

around 100 mg, and after immersion, their mass was monitored throughout the days to evaluate the water intake.

The first swelling test was performed with sample 5.A, and the next with sample 7.A. Both samples were identical in terms of synthesis (Type I) and quantities of used compounds. Two films of each sample was prepared, and in Figure 3.5 is showed the average change in mass over the course of 8 days.

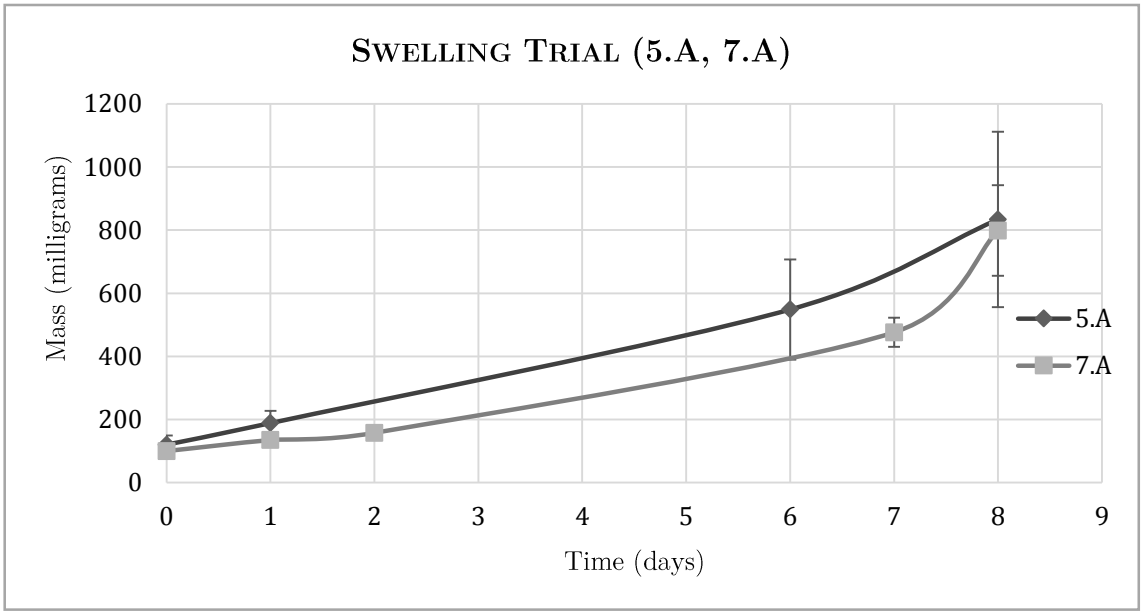

Figure 3.5 – Swelling behaviour of samples 5.A and 7.A over the course of 8 days.

By analysing the results, both samples had a rapid water intake over the days, increasing in mass and in volume. After six days, the films from sample 5.A started to display big discrepancies in mass, explicit in the error. By the eighth day, the films started to degrade, and concerning sample 5.A, there was once again a large discrepancy in mass, being the average value 833.9 ± 277.9 mg. In sample 7.C, the average mass was 798 ± 143.8 mg after eight days. This rapid swelling was not the intended, since in order to get a better drug releasing profile, the water intake must be slow and the films should maintain integrity for longer time when immersed in the solution.

As mentioned in Chapter 1, a higher cross-linking degree of the hydrogel decreases the water intake, lowering the swelling ratio and slowing down the

process. To overcome this, samples from Type III synthesis were tested for swelling, since BTCA and Bis-MPA could provide a higher cross-linking degree to the hydrogels. At first, a trial with BTCA was performed with four films created with sample 1.C, and the average swelling behaviour is displayed in Figure 3.6.

By looking at the swelling plot of sample 1.C, despite the rate of water intake is similar in first day, it is clear that there was a significant delay in the swelling over the subsequent days, since the mass of the films had a smaller increase, when comparing to samples 1.A and 7.A. Taking in example the eighth day, when the samples 5.A and 7.A started to degrade and had swollen greatly – in this case the mass was 195.5 ± 15.4 mg, roughly twice the initial mass. With this results, the introduction of BTCA produces a material with a lower swelling degree and a longer durability, since it started to degrade only after day 25 after immersion, and so, it was decided to be target for further studies.

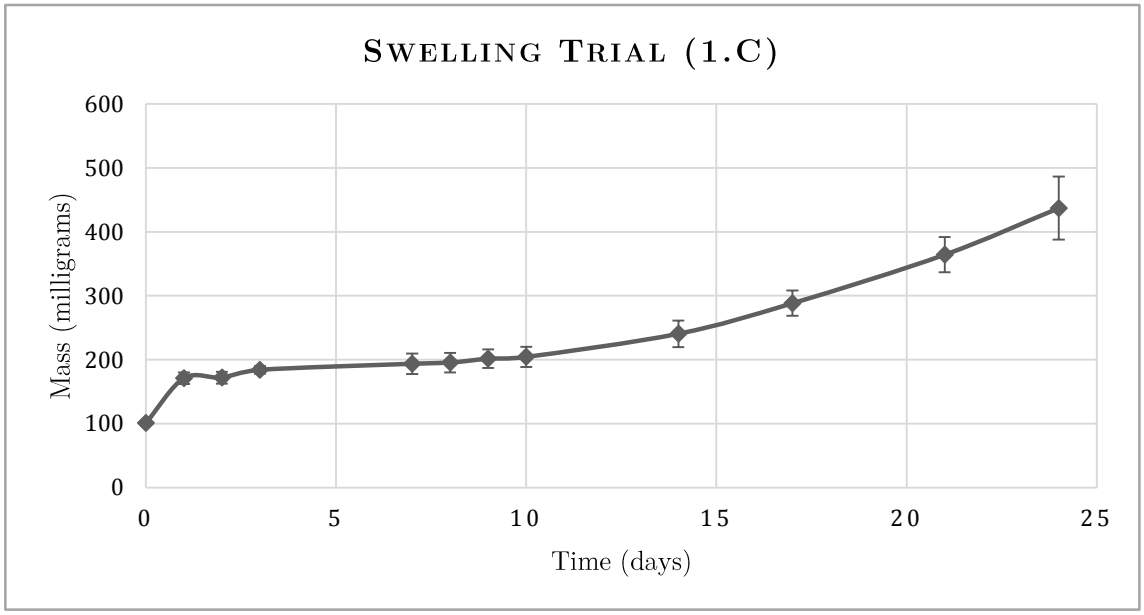

Figure 3.6 – Swelling behaviour of sample 1.C over the course of 24 days.

Three samples with different amounts of BTCA were prepared, and subsequently four films of each sample were made and their swelling behaviour was monitored. These samples correspond to samples 2.C, 3.C and 4.C, and each

comprised 25, 50 and 100 mg of BTCA, respectively. The swelling behaviour of these samples is presented on the following Figure 3.7.

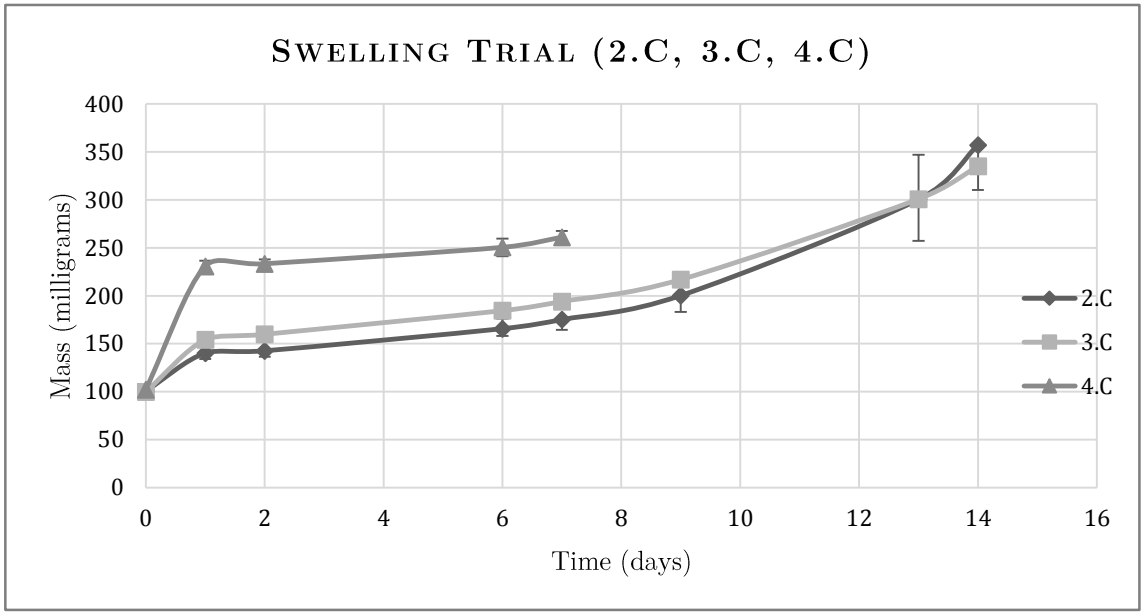

Figure 3.7 – Swelling behaviour of samples 2.C, 3.C and 4.C over the course of 14 days.

By analysing the results, samples 2.C and 3.C had a relatively similar behaviour over the course of the 14 days. Sample 4.C had a higher mass increment over the first day (taking approximately 11 days for samples 2.C and 3.C to reach), and on the seventh day, since the films started to show a fragile appearance, they were discarded, and for that reason, only samples 2.C and 3.C were monitored over the next days. Since both 2.C and 3.C had similar behaviour and properties, and since both had 25 and 50 mg of BTCA, respectively, it was decided to proceed with 30 mg of BTCA in the synthesis for the further drug encapsulation, since a trial was made as well and it presented similar properties to the samples 2.C and 3.C presented in Figure 3.7.

In the meantime, a trial with Bis-MPA as additional compound was performed (sample 5.C). Therefore, four films were made with sample 5.C and submerged into 8 ml of saline solution to monitor the change in mass over the days. The plot is displayed in Figure 3.8.

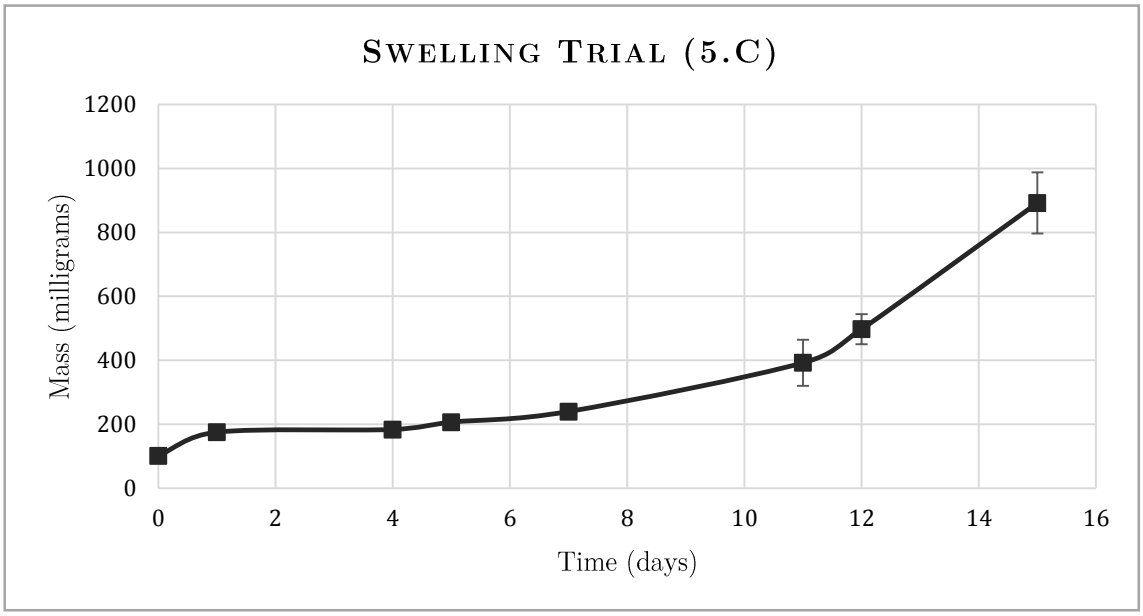

Figure 3.8 – Swelling behaviour of sample 5.C over the course of 15 days.

Observing the results, the mass almost doubled during the first day, but then it stabilized to a particularly slow swelling rate until day 7. After this point, the films started to swell at a slightly faster rate, and the final monitored mass was of about 891.9 ± 95.6 mg. Even though the water intake of these films was faster and higher than BTCA, it showed an appropriate swelling profile for the intended purposes and it was decided to include Bis-MPA in the sample preparation for the further drug encapsulation.

3.2.2 Drug Releasing Tests

Once the study of the swelling degree and behaviour of the different compounds was performed, the selected samples for drug encapsulation were 8.C, 12.C and 16.C, with BTCA as additional compound, and the samples 9.C, 13.C and 17.C with Bis-MPA as additional compound, since the samples prepared by

Type III were the most promising regarding the swelling rate and degradability. The study was carried out in pairs of two samples, one with BTCA and one with Bis-MPA, specifically, 8.C and 9.C, 12.C and 13.C, together prepared using Encapsulation Method I and 16.C and 17.C, prepared using Encapsulation Method II.

Encapsulation Method I

This method refers to samples 8.C, 9.C, 12.C and 13.C, and concerns the formation of a film with the 5- Fu as a model drug. For the preparation of the film, 100 mg of dried sample powder were mixed with 6 mg of 5-Fu and submitted to a press. After preparation, the films were submerged in 8 ml of saline solution and kept inside an incubator at 37^{o}C for the release to occur, being the absorbance measured 4 hours after immersion and in the following days as well.

Samples 8.C and 9.C were a first trial for the drug releasing, and for this reason, the results for these two samples will not be presented.

Concerning samples 12.C (with BTCA) and 13.C (with Bis-MPA), they followed the same method as 8.C and 9.C, where 100 mg of sample powder were mixed with 6 mg of 5-Fu and submitted to a press in order to form the film with the encapsulated drug. In total, four films were created for each sample, one without the drug, for reference, and three with the drug encapsulated. The absorbance was measured after 4 hours of immersion and in the following days as well, making the process go on for 20 days. The amount of released drug was determined using the calibration curve prepared beforehand, mentioned in Chapter 2 in section 2.3.5. The concentration of 5-Fu in the saline solution was settled by relating it to the measured absorbance through Equation 2.1, and, by knowing the operating volume, it was possible to calculate the amount of released 5-Fu.

The results of the average release for sample 12.C and 13.C follow in Figure 3.9, and is presented the percentage of the initial mass of 6 mg that was released

to the aqueous solution (100% is equivalent the total 6 mg). Both samples showed a fairly similar behaviour during the progression of the 20 days. It is clear that a prompt release takes place during the first 4 hours, being that 80.0 ± 5.5% of the drug was released to the saline solution by sample 12.C and 76.5 ± 4.9% by sample 13.C. After this stage, the concentration of the drug barely varied, since the final value after 20 days was 85.5 ± 7.7% for sample 12.C and 81.6 ± 7.9% for sample 13.C.

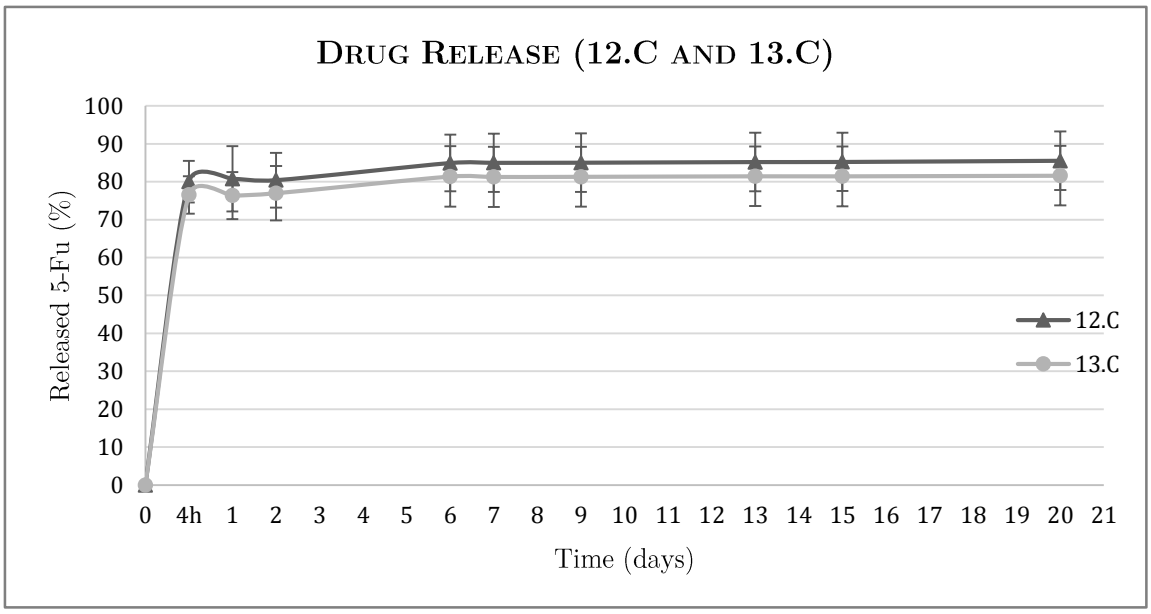

Figure 3.9 – Comparison between the 5-Fu releasing profiles by samples 12.C and 13.C over the course of 20 days.

By comparing the release between samples 12.C and 13.C during the course of the 20 days, it is visible that sample 13.C shows a slightly lower release, but taking in account the error, this difference is not significant. The fast release can be attributed to a different releasing system other than a swelling controlled release, noticeably showing that the drug would dissolve into the saline solution by direct diffusion, instead of swelling related diffusion. In short, the drug would be in direct contact with the surrounding fluid, causing it to dissolve before the major swelling of the film occurred. Since the initial purpose was to create a swelling controlled releasing system, this shortcoming had to be improved and an attempt to do it is presented in Encapsulation Method II.

Still concerning sample 13.C, Figure 3.10 displays an image of a swollen film after 25 days, showing a gel-like consistency and an increase in volume and mass.

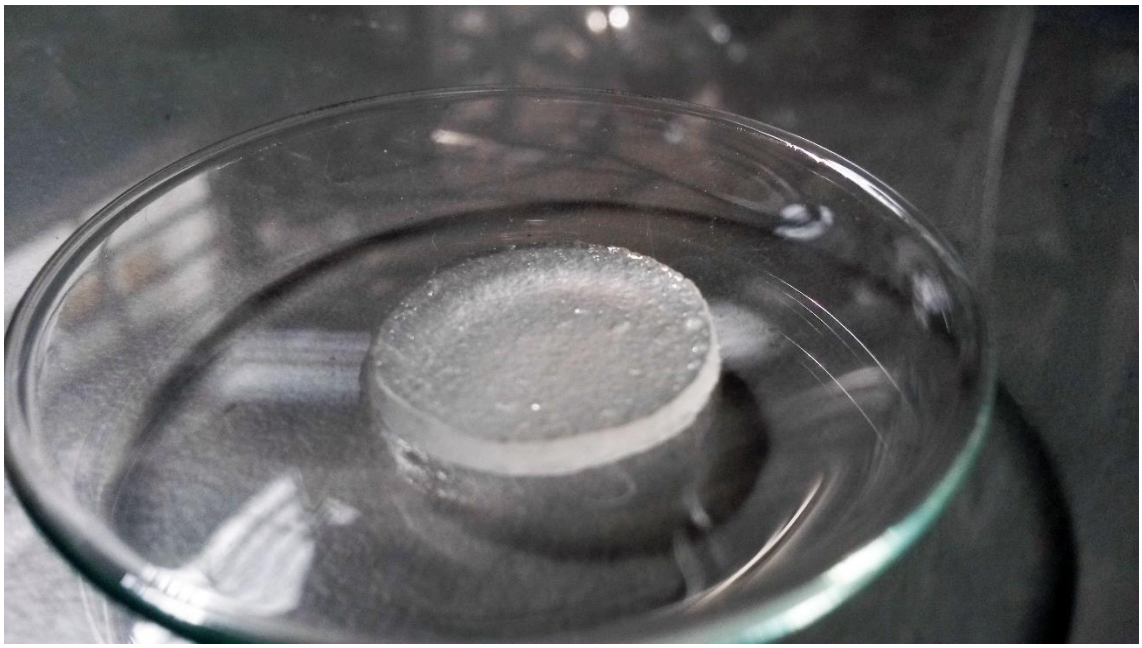

Figure 3.10 – Swollen film of sample 13.C after 25 days of immersion.

To evaluate the surface properties of the films, SEM imaging was performed. For this, the films without 5-Fu, made for reference with samples 12.C and 13.C, were lyophilised after being immersed in saline solution for 10 days. Two of the obtained images follow in Figures 3.11 and 3.12.

Figure 3.11 concerns sample 12.C and shows large cracks across the surface. Those cracks can possibly confirm the fact that the drug would be released by diffusion in the first hours, since the surrounding fluid could penetrate the film through those cracks, allowing the drug to be dissolved before the swelling of the film occurred. In sample 13.C, similar cracks were visible as well. In figure 3.12, concerning sample 13.C, a large crack also appears, however, in this particular case, a wave-like pattern emerges on the surface. Perhaps, this pattern can be attributed to the swelling behaviour of the film.

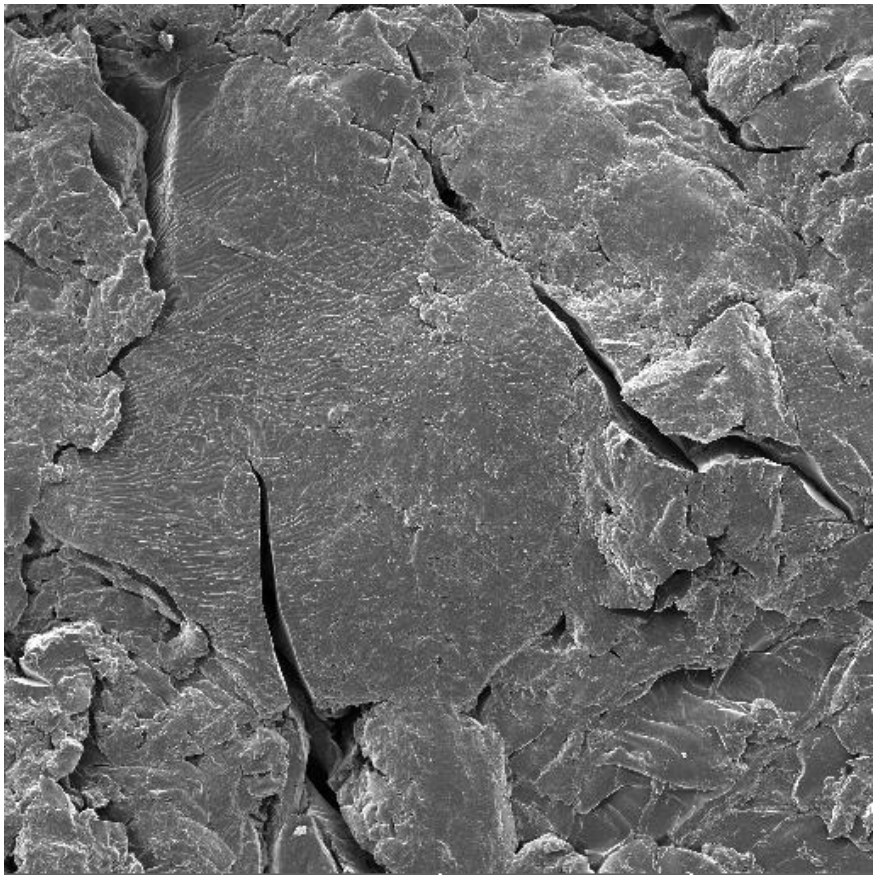

Figure 3.11 – SEM image of sample 12.C with a magnification of 200x

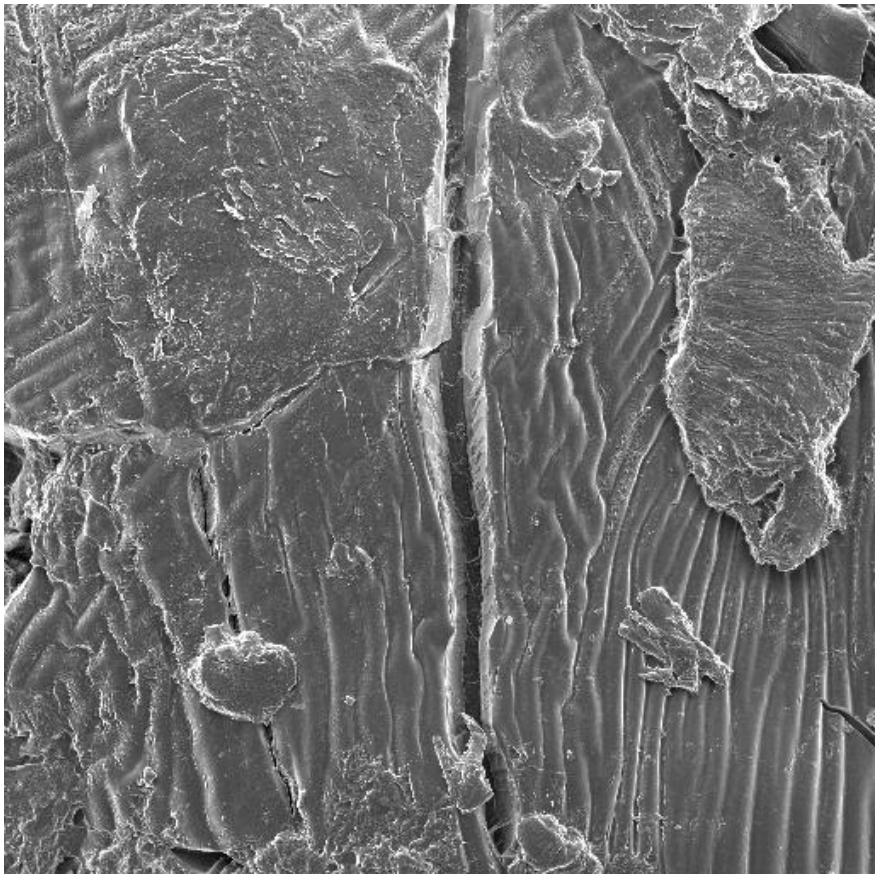

Figure 3.12 – SEM image of sample 13.C with a magnification of 503x

Encapsulation Method II

Since the drug releasing tests using films created by Encapsulation Method I showed an extremely high releasing profile during the first 4 hours, a way to overcome this flaw had to be developed. For this reason, an upgrade to Encapsulation Method I was made by laying a hydrophobic coating onto the films with a $CHCl_3$ solution of polyester and PLA (50/50). This attempt was made with purpose of creating a barrier between the film and the solution in which the release would happen, in order to prevent a direct diffusion of 5-Fu and delay the fast release profile during the first hours. The primary segment of the process was similar as in beforehand, and four films of about 100 mg of each sample 16.C (with BTCA) and 17.C (with Bis-MPA) were made – one without the drug, for reference, and three others comprising 6 mg of 5-Fu. In advance, the films were immersed into the polyester-PLA solution to ensure the coating and left to dry at room temperature for a few minutes. After this, the films were submerged into 8 ml of saline solution and kept into an incubator at 37^{o}C for the release to occur. The absorbance of the solution was, once again, measured 4 hours after immersion and in the following days, making the monitoring of the experiment last for 10 days.

The results concerning samples 16.C and 17.C follow in Figure 3.13, plotting the drug released to the saline solution through the days by both samples.

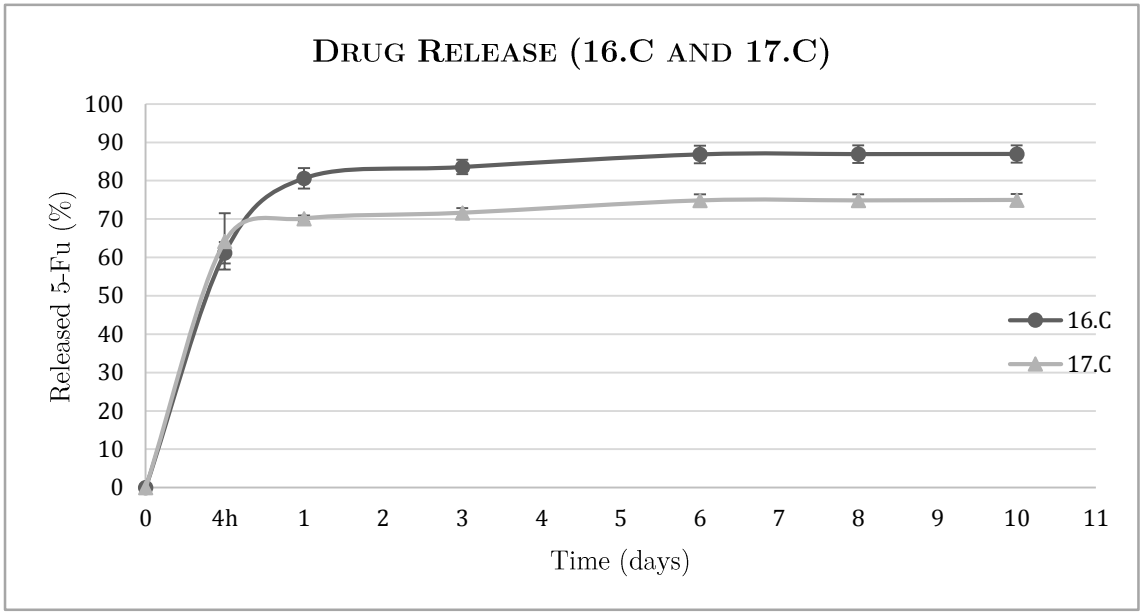

Figure 3.13 – Comparison between the 5-Fu releasing profiles by samples 16.C and 17.C over the course of 10 days.

Observing the results of the release by these two samples, it is visible that there was a slight decrease in the first 4 hours, when comparing to the tests ran with samples 12.C and 13.C, and this fact is most probably due to the coating present in the films. In spite of this reduction, the values after 4 hours remained higher than it would be expected, since the values were 61.2 ± 2.8% for sample 16.C and 64.2 ± 7.3% for sample 17.C. After the first day, the amount of 5-Fu in the saline solution rose to 80.6 ± 2.7% in sample 16.C and to 70.1 ± 0.8% in sample 17.C. Comparing the releasing profiles of both samples 16.C and 17.C, they show a similar profile, but with the sample 17.C having a lower release percentage, the same way as it happened among samples 12.C and 13.C. The drug amount in solution remained relatively constant after the first day, and after 10 days the total release was 87.0 ± 2.3% for sample 16.C and 75.0 ± 1.6% for sample 17.C, making the release profile fairly similar as the one monitored before with samples 12.C and 13.C.

By examining the films after one day, it was noticeable that the coating was starting to peel off on the sides of the films, allowing the solution to penetrate and be in direct contact with the films, causing the 5-Fu to dissolve directly by diffusion and triggering the same drawback as in the Encapsulation Method I, and this is visible in Figure 3.14.

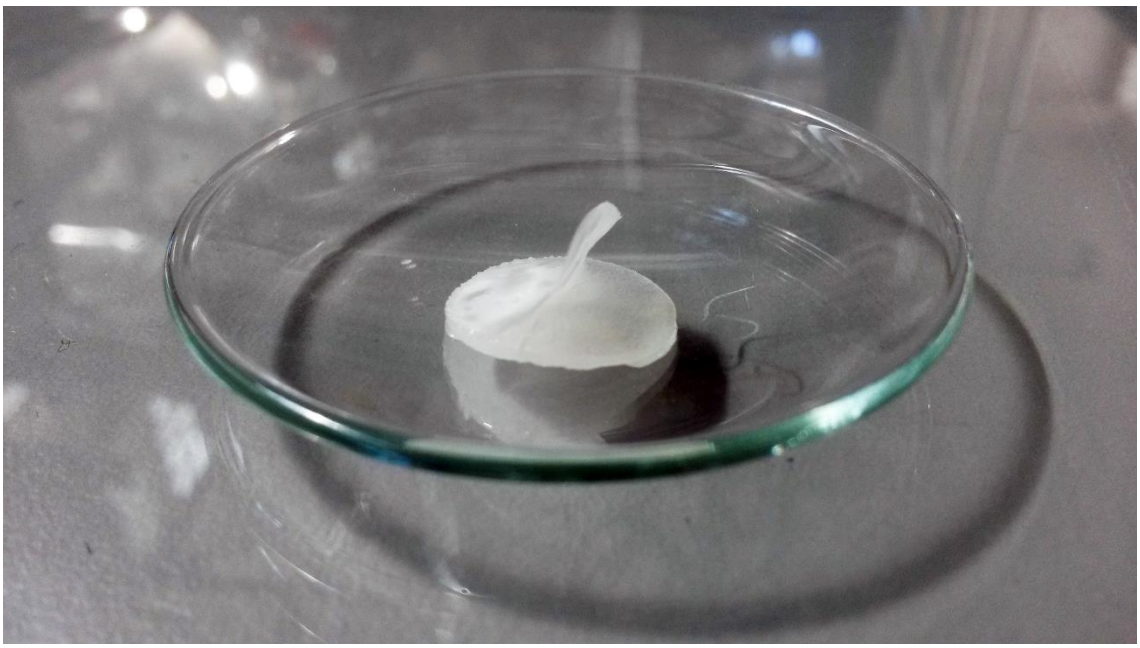

Figure 3.14 – Image illustrating the lack of adhesion of the coating on sample 17.C.

The results in both of the methods of encapsulation displayed a profile that deviates from what would be expected, since most of the drug is released within the first four hours, and in an ideal drug delivery system, a consistent and somewhat constant release has to be attained. In Figure 3.15 follows a comparison between all the fours samples subjected to releasing tests for a period of 10 days, demonstrating a fairly similar profile in a long term, only showing slightly substantial differences during the first 4 hours. Compounds with Bis-MPA in its synthesis showed a slightly lower releasing profile over the days.

The differences between the pairs 12.C/13.C and 16.C/17.C can be attributed to the inclusion of the polyester-PLA coating on the later, but since the

coating did not adhere properly to the films, after the first day the behaviour becomes fairly similar.

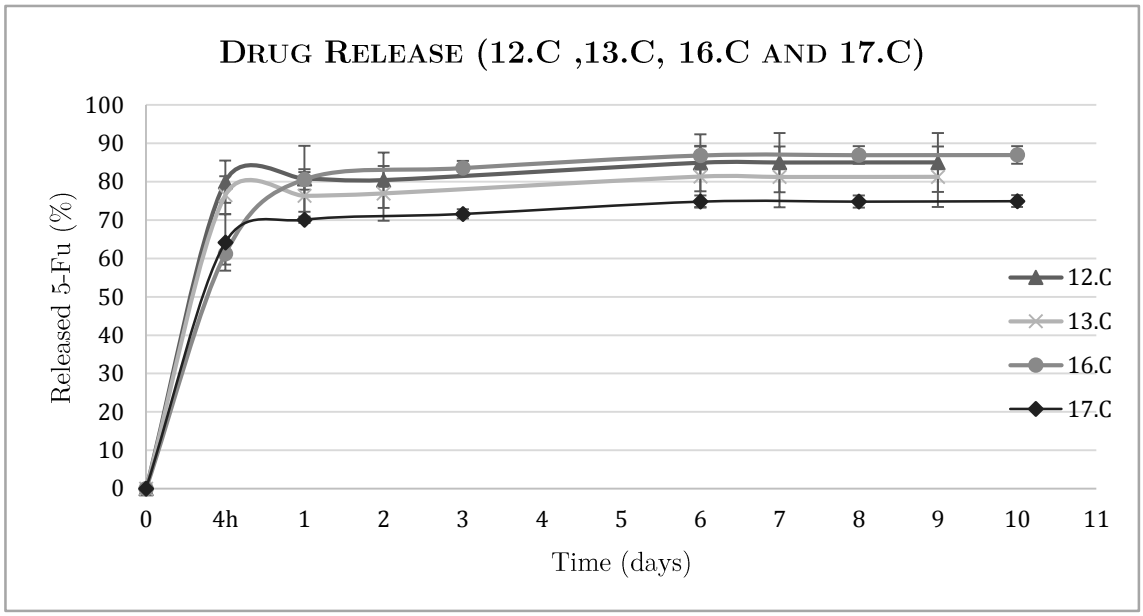

Figure 3.15 – Comparison of the 5-Fu releasing profiles by samples 12.C, 13.C, 16.C and 17.C.

Concerning the samples 12.C and 13.C in the drug releasing trials, an attempt to replace the full volume of the surrounding solution for a new one was made on day 3 of immersion, and in the case of samples 16.C and 17.C the replacement occurred on day 4. The amount of 5-Fu released afterwards was added to the total amount that was released until these instants, thus becoming cumulative results hereafter. In the case of samples 12.C and 13.C, the values were 80.4 ± 7.2 % and 77.0 ± 7.1 % before the replacement, respectively, and 85.5 ± 7.7 % and 81.6 ± 7.9 % on the final day, respectively. In case of samples 16.C and 17.C, the values were 83.7 ± 1.9 % and 71.6 ± 1.2 % before the replacement, respectively, and 87.0 ± 2.3 % and 75.0 ± 1.6 % on the final day, respectively. By evaluating these results, we are able to conclude that although the samples were in contact with a new medium, the replacement did not bring a considerable difference in the amount of released 5-Fu, since the values did not show a significant increase in the release. This demonstrates that the remaining 5-Fu (until the 100%) was not

being released due to a saturation of the surrounding medium, but because it was trapped inside the films and was not easily released. Perhaps this remaining 5-Fu would only be released after a much more advanced swelling of the films.

One goal of the work was to relate the drug release to the swelling rate of the films, as in a swelling-controlled releasing device. It was verified that a swelling-controlled release did not occur due to the fact that most of the drug was released to the surrounding fluid long before the films even began to swell. The Encapsulation Method II was implemented in an attempt to inhibit/reduce this type of behaviour, but since the coating did not adhere very well, this attempt was failed – even so, the releasing profile was lowered during the first 4 hours. Due to a lack of time in the end of the studies, it was not possible to develop an encapsulation method to overcome this deficiency, but alternative proposals will be discussed in the future prospects present in Chapter 4.

In literature one can find several drug releasing methods for 5-Fu, and although these studies are not directly related to the same hydrogels as in this study, they supply some information for comparison with the obtained results. For instance, Lin & Fu (2009) presented chitosan/polyethylene glycol microparticles loaded with 5-Fu for a controlled release and show cumulative results for this releasing. The releasing profiles presented by Ling & Fu are monitored for 44 hours and they display a cumulative release for the first 4 hours between 10 and 30% in several cases, showing a significantly less percentage during the same period when comparing to the results obtained by the presented study, in which the results were between around 60 to 80%. [44]

Li *et al.* (2013) presented chitosan-based graft copolymer cross-linked hollow spheres sensitive to temperature and pH for 5-Fu release, and display cumulative releases at 37°C and pH 7.4 of around 25% after 4 hours, 60% after 10 hours and 80% around 50 hours, once again showing an improvement when

compared to the drug releasing tests performed in this study, which, as demonstrated before, showed releases between 60 and 80% after 4 hours. [45]

McCarron *et al.* (2000) prepared polymeric nanoparticles for a 5-Fu sustained release with several materials and display the releasing profiles monitored for 24 hours. The nanoparticles of the various materials presented especially rapid releases after 4 hours in between 65 and 85%, and after that, the releasing profile was essentially stabilized until the final 24 hours. This particular case is more analogous to the obtained results by this study, showing similar releasing profiles over the first day. [46]

3.3 FTIR Analysis

In this section are presented the spectra obtained from the samples by FTIR ATR analysis to be evaluated and compared in a qualitative way.

The cross-linking process was attained by bonding together the functional groups of the cross-linking agent X to the hydroxyl groups present in dextran, in concern of Type I synthesis. In Type II and Type III synthesis, the cross-linking process occurred by bonding the functional groups of the cross-linking agent X to the hydroxyl groups present in dextran and to the carboxyl and hydroxyl groups present in the additional cross-linking agents added to the synthesis. This bonding is characterized by the presence of carboxyl groups and, customarily, this carbonyl stretch (C=O) appears in a band comprised between 1760 and 1690 cm^{-1} in the FTIR spectrum.

In the prepared samples, aside from the amplitude variances, the major difference in the spectra when comparing with the spectrum of unmodified dextran is the appearance of a peak comprised around 1740-1760 cm^{-1}. This peak is comprised in the carbonyl stretch band and confirms the occurrence of the cross-linking, and this is present in all the evaluated samples.

In Figure 3.16 follows the FTIR spectra of samples 5.A and 7.A (analogous in terms of synthesis) to compare with the spectrum of dextran. The spectra are quite similar, and the major difference is in the formation of two peaks around 1750 and 1805 cm^{-1}, possible attributed to the formation of the cross-linking process.

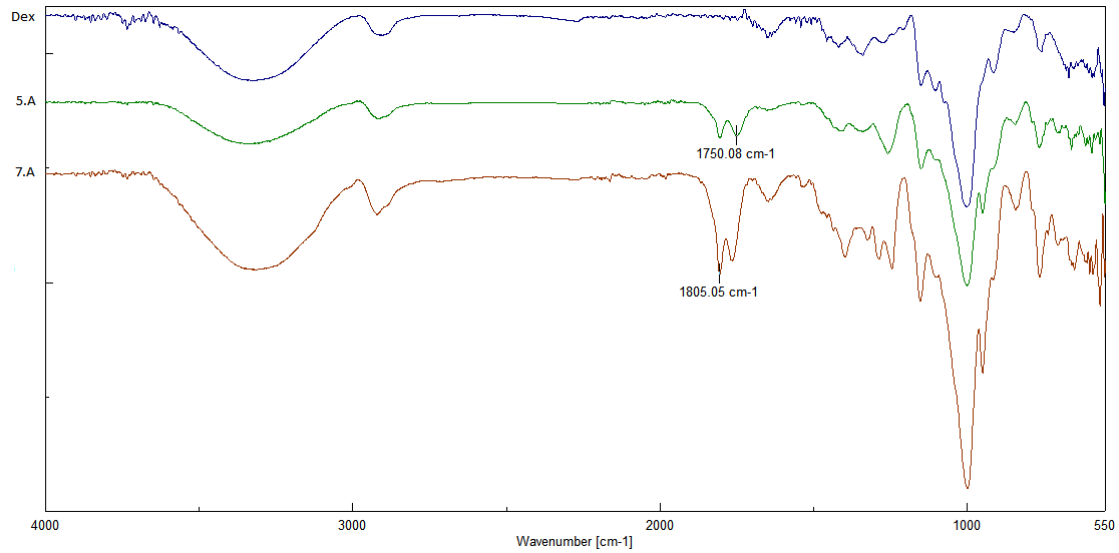

Figure 3.16 – FTIR spectra of samples 5.A, 7.A and simple dextran.

In Figure 3.17 are presented the spectra of samples 4.B, 5.B and simple dextran. Once again, the major difference when comparing the obtained samples with simple dextran is shown to be in the formation of a peak around 1740 cm^{-1}, comprised in the carbonyl stretch and can be attributed to the cross-linking process.

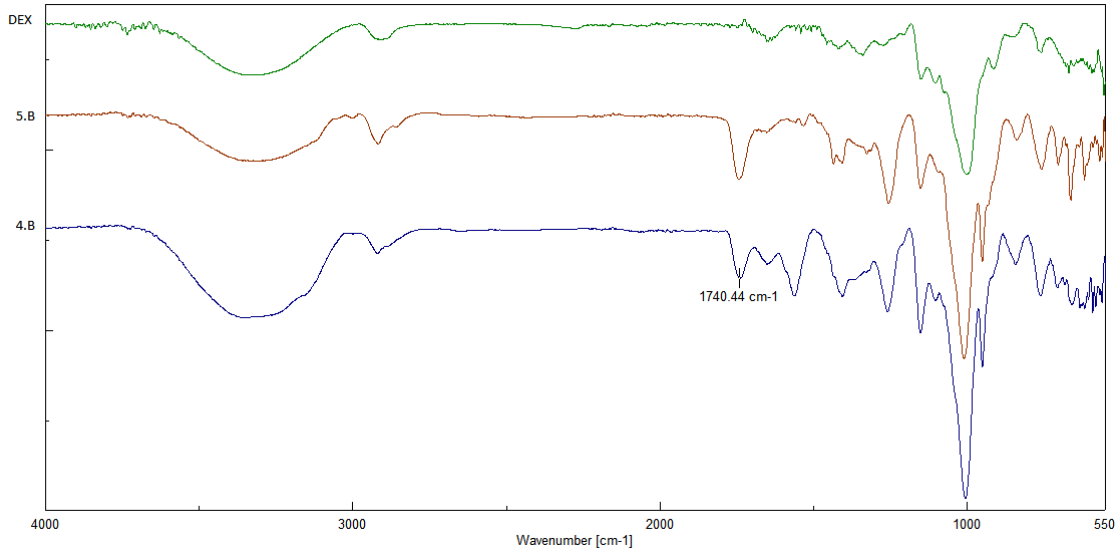

Figure 3.17 – FTIR spectra of samples 4.B (with succinic acid), 5.B (with sebacic acid) and simple dextran.

In Figure 3.18 are presented the spectra of samples resulting from Type III synthesis to compare with simple dextran, precisely samples 2.C (with BTCA) and 17.C (with Bis-MPA). The major differences are once again in the formation of two peaks around 1751 and 1804 cm^{-1}, attributed to the cross-linking process.

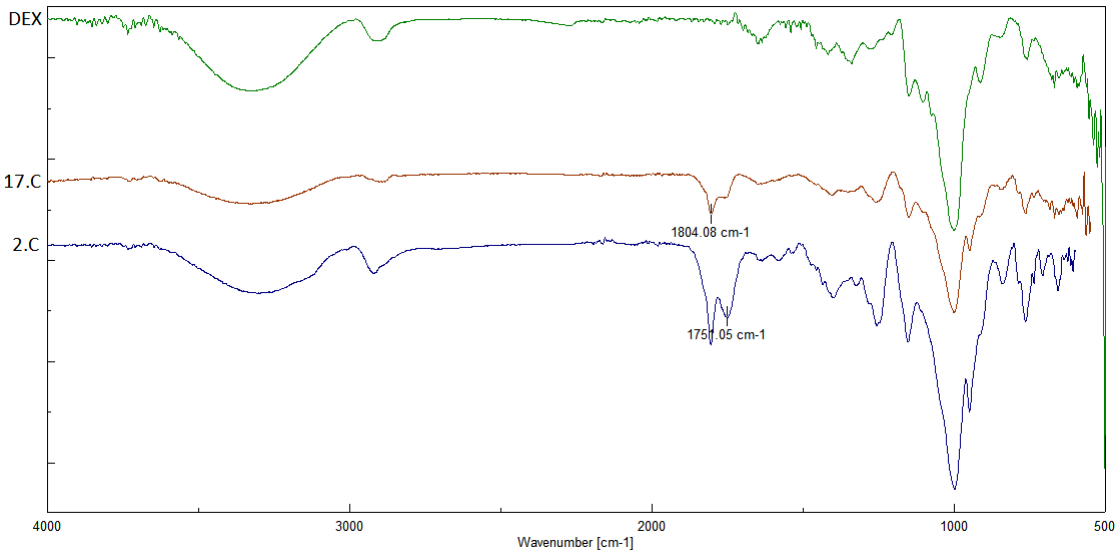

Figure 3.18 – FTIR spectra of samples 2.C (with BTCA), 17.C (with Bis-MPA) and simple dextran.

4 Conclusions and Future Work

4.1 Conclusions

Concerning the present study, hydrogels were prepared and studied in order to find suitable materials for a drug releasing system. The used polymer was dextran with a molecular weight of 450.000-650.000 Da, and by reaction with a cross-linking agent it originates a gel like material. Different compounds were added to the initial formulation in order to improve the cross-linking properties of the prepared materials through their hydroxyl and carboxyl groups. Three types of synthesis were performed. Type I synthesis comprised only dextran and the cross-linking agent X in the synthesis, and several of the prepared compounds were not suitable for film formation. In Type II synthesis, additional compounds were added as secondary cross-linking agent, in their majority dicarboxylic acids. Samples prepared by Type II synthesis were found to have undesirable properties. Concerning Type III synthesis, dextran hydrogels comprising BTCA and Bis-MPA in their structures were found to have suitable swelling profiles and were carried out to the drug encapsulation stage.

An apparent major concern relating to the film formation was found in the majority of the samples prepared by Types I and II synthesis, and in a small number of cases by Type III. Some samples did not form films at all, and in other cases the

film was achieved, but did not attain suitable properties. The reason for this could be related to a poor DMSO removal in 2-propanol after the synthesis of the hydrogels.

Concerning the drug releasing trials, the Encapsulation Method I showed an extremely high 5-Fu release during the first 4 hours. There was an attempt to overcome this issue with the Encapsulation Method II, by coating the films with a PLA-polyester solution to decrease the release during the first hours. In this case the release was lowered to some extent, but still retained a prominent profile during the first hours. After the first day, the coating began to peel out of the films, and the release reached similar levels as in Encapsulation Method I. An ideal drug releasing method would release the 5-Fu proportionally to the swelling of the films, with a slow and relatively constant profile until the degradation of the film, but in both of the encapsulation methods this was not achieved. SEM imaging of some samples demonstrated cracks in the films, which probably allowed the surrounding fluid to penetrate the films and dissolve the 5-Fu. Therefore, the drug releasing happened in terms of direct diffusion, instead of a swelling related diffusion, since the majority of the drug was released before the films were swollen. The encapsulation method of the drug comprising the mixture of 5-Fu directly with the polymer powder before film formation, instigates that the drug would not be homogeneously distributed throughout the films, and some of the drug probably remained at the surface of the films, thus assisting its direct diffusion.

With this, it is clearer that the encapsulation method was not properly correlated with the properties of the prepared materials, since did not take advantage of the good swelling profiles attained by some samples, especially those resulting from Type III synthesis.

One of the main goals of this work was to improve the cross-linking properties of the modified dextran in order to delay the swelling ratio and

consequently delay the drug releasing, and the improvement in terms of swelling was, in fact, achieved. However, it is clearer now that a suitable profile is not achieved by limiting the speed of access to the interior of the films (by increasing the cross-linking) but instead by limiting the access to the interior of the films (by resorting to different encapsulation methods). The coating employed in Encapsulation Method II showed a slight improvement to this matter, since it limited the access of the surrounding fluid to the interior of the films, even if it was for a short time and in a somewhat ineffective way.

In summary, the prepared work was advantageous to the extent of preparing suitable biodegradable materials for drug encapsulation, however, the drug encapsulation did not show successful and displayed unpleasant results when comparing to the expectations. On the other hand, the swelling behaviour of the prepared materials using BTCA and Bis-MPA as additional compounds revealed appealing results.

4.2 Future work

Throughout the swelling tests of the prepared materials, some samples displayed a suitable swelling profile and degradation for a sustained drug releasing profile, specifically samples from Type III synthesis, employing BTCA and Bis-MPA as additional cross-linking agents. Since the drug releasing studies were not successful and did not correlate to these properties of the materials, new methods to encapsulate the drug have to be developed in order to overcome the fast releasing profile during the first hours. Understanding the fact that the included coating in Encapsulation Method II did improve the releasing profile during the first hours and that it began to peel off after the first day of immersion, it is clear that if an improvement in the adherence of the coating was attained, the rate of drug release would reduce, improving the releasing profile over the days.

Even with an improved coating, the method of dispersion of the drug would not be homogeneous if the drug is mixed directly with the polymer powder, thus arising problems in consistency. Therefore, an enhanced method of including the drug inside the films needs to be developed. Alternatives can be achieved by including a drug reservoir inside the films – by this method, the drug would not be in direct contact with the surrounding fluid until the swelling of the hydrogel allowed the water to penetrate and become in contact with the drug reservoir. A less undemanding alternative can be attained by creating a 'sandwich-like' film. Instead of mixing the drug directly with the full mass of the film before submitting it to a press, a layer method can be performed. For instance, 50 mg of sample can be deposited in the bottom, thus creating a base, then, a mixture between 50 mg of the same sample and an amount of 5-Fu would be deposited over the base, and this 'sandwich-like' film would be topped with more 50 mg of sample. If necessary, a similar coating as the one employed in the studies might be appropriate.

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